Supporting Information (SI)

Hydrophilic Small Molecules that Harness Transthyretin to Enhance the Safety and Efficacy of Targeted Chemotherapeutic Agents

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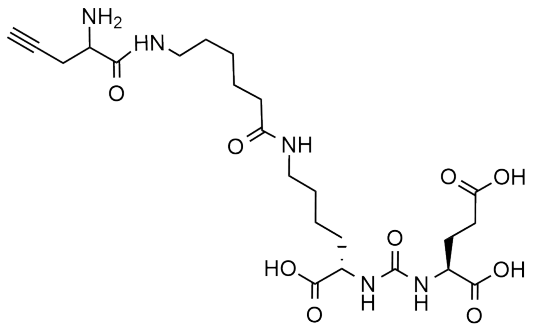
Supporting Figures



Ligand **22**

Figure S1. Chemical structure of PSMA ligand **22.** Ligand **22** is a spacer-modified version of a known glutamate-urea-lysine PSMA ligand.

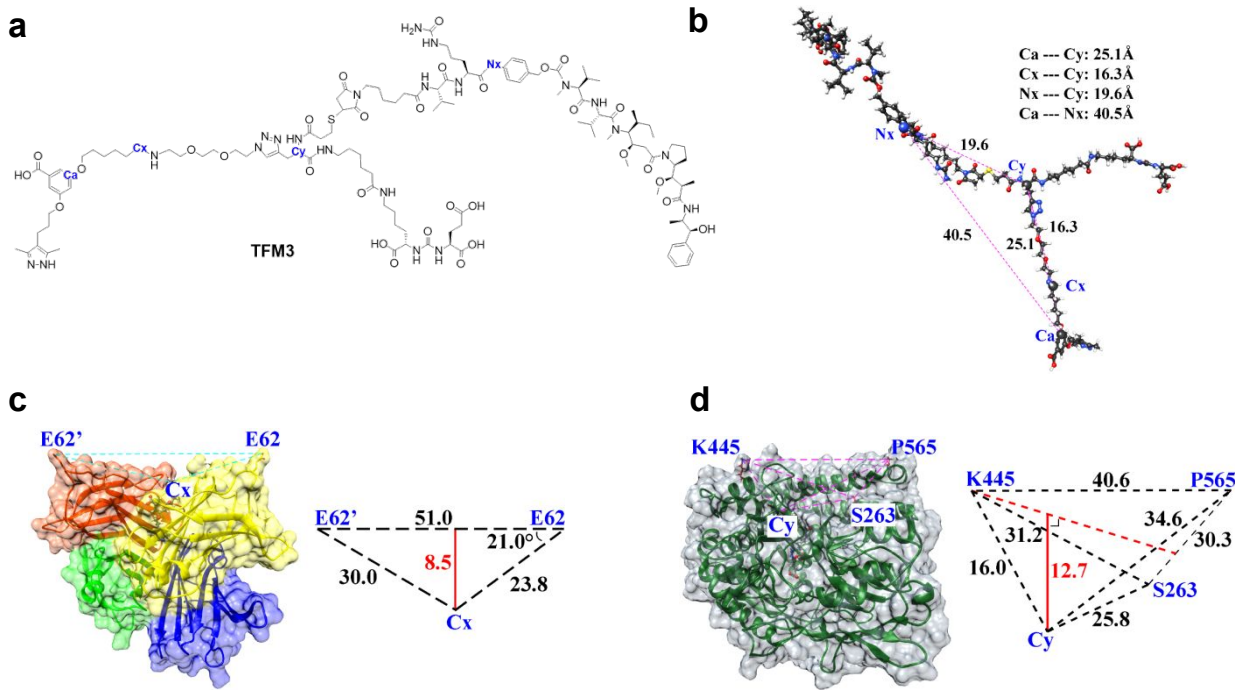


Figure S2. Modeling studies of TFM3 with both TTR and PSMA to determine the appropriate linker lengths. (a) Chemical structure of TFM3 highlighting the carbons (Ca, Cx, and Cy) and nitrogen (Nx) atoms used for measurement of distance in the modeling. (b) Structure of an optimized conformation of TFM3. Atom labels and the distances of interest are shown. All the distances are in Å. (c) We used the TTR:AG10 crystal structure (pdb id: 4HIQ)[1] for TTR binding site measurement. Two glutamine residues from chain A (E62) and chain A' (E62') are two residues highest from the center of the binding site. Cx is the terminal carbon atom in the TTR ligand **2**, which is at the highest position from the bottom of the binding pocket. The scheme to the right shows the measured distances between oxygen atoms on E62 and E62', and Cx atom. The distances are given in Å and the angle Cx-E62-E62' is 21.0°. The distance from Cx to the surface of the TTR is at least 8.5 Å. (d) We used the crystal structure of PSMA (pdb id: 2XEF)[2] for PSMA binding site measurement. The entrance of binding site is very wide and broad. The highest three residues surrounding the entrance are identified as serine 263 (S263), lysine 445 (K445) and proline 565 (P565). Position of Cy atom in the docked known glutamate-urea-lysine ligand for targeting PSMA was used to measure the distance to the surface of PSMA. The scheme to the right shows the distances between S263, K445, P565, and Cy atom. The distance from Cy to the surface of PSMA was found to be minimum 12.7 Å. In our linker system, the Cx-Cy distance is ~16 Å, which is too short to bring the two proteins in close proximity to each other. A linker of at least 21 Å (8.5 Å + 12.7 Å) is required to form the ternary complex between TTR, PSMA, and TFM3.

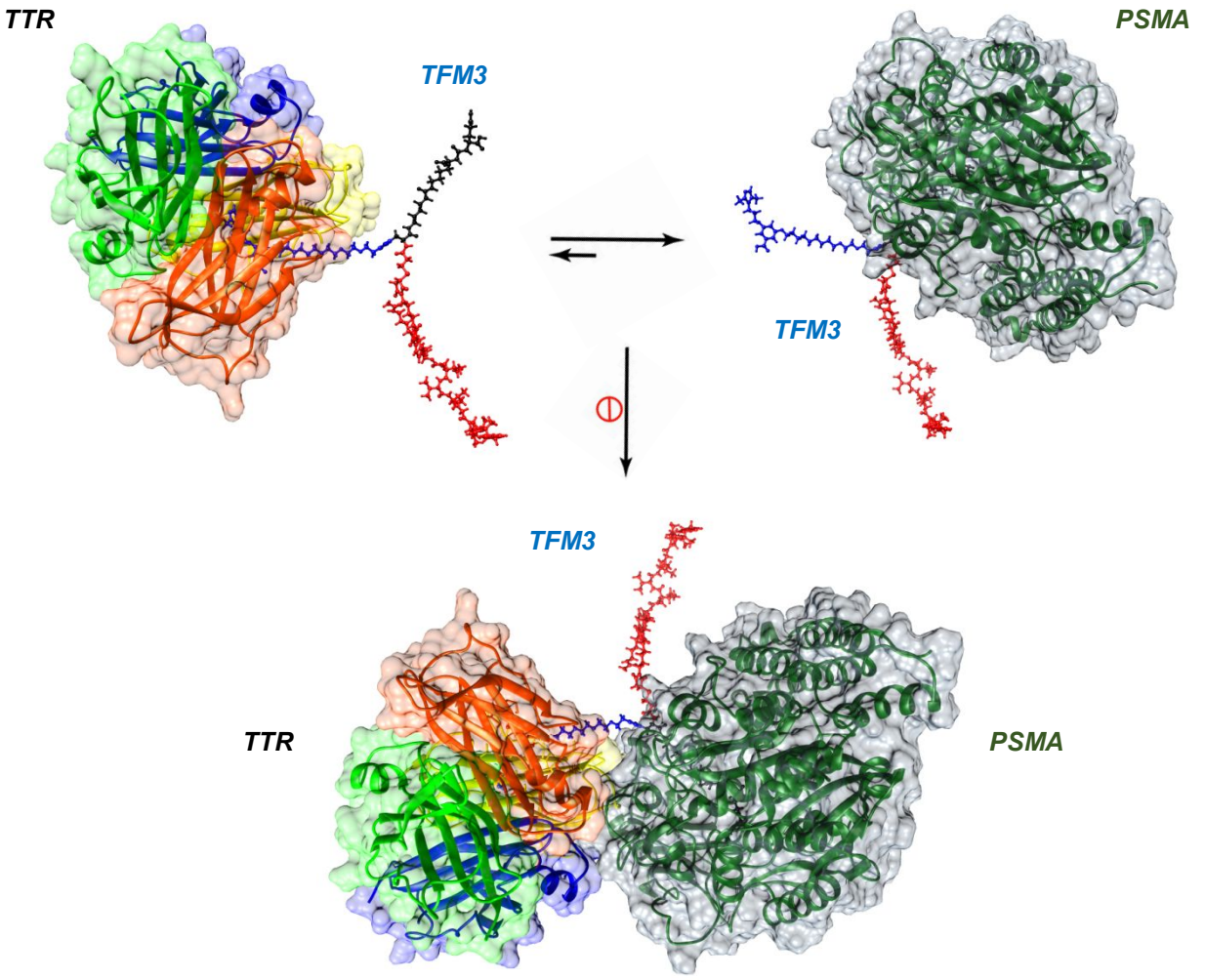


Figure S3. Modeling studies of TFM3 with both TTR and PSMA suggested that the linker we used (~16 Å) is too short to bring the two proteins in close proximity to each other. A linker of at least 21 Å is required to form ternary complex between TTR, PSMA, and TFM3. Schematic binding of TFM3 with TTR and PSMA. TFM3 was super imposed onto ligand **2** with TTR and onto the glutamate-urea-lysine ligand **22** docked with PSMA. Hypothetical complex of TFM3 with TTR and PSMA is shown at the bottom of the figure. Due to the short distance, this formation of the ternary complex between TFM3, TTR and PSMA is not favored.

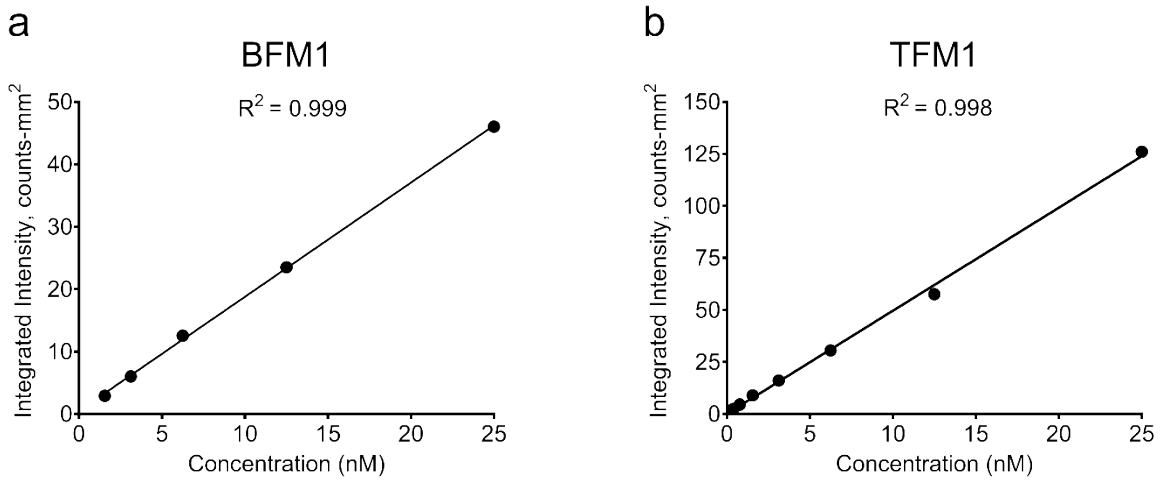


Figure S4. Calibration curves used to quantitate (a) BFM1 and (b) TFM1 in rat plasma. The data were generated using LI-COR Odyssey® CLx Imaging System.

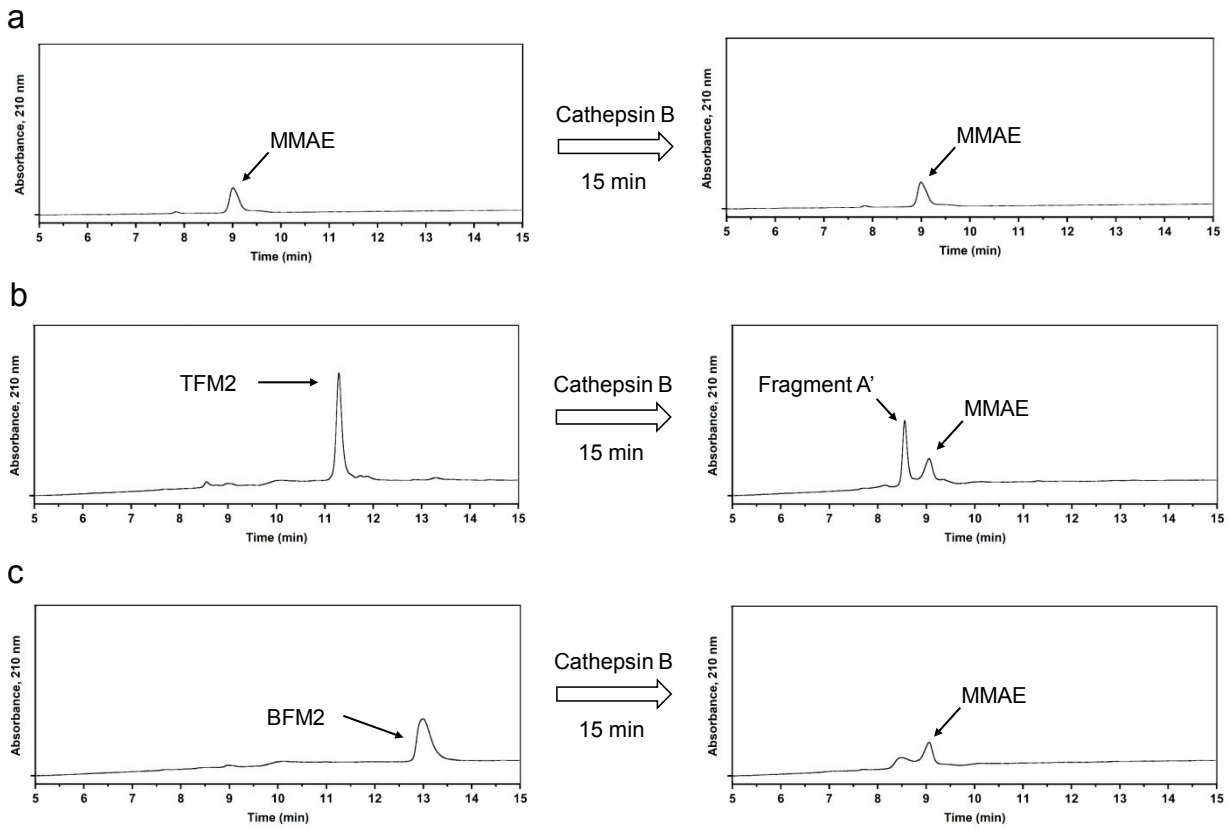


Figure S5. MMAE release from TFM2 and BFM2 upon cathepsin B cleavage. (a) HPLC trace of free MMAE showing that no hydrolysis occurs by cathepsin B. (b-c) The valine-citrulline dipeptide cleavable linker in TFM2 and BFM2 is efficiently cleaved (within 15 min in buffer) by cathepsin B hydrolysis and spontaneous fragmentation of the p-aminobenzylcarbamate intermediate. The formation of free MMAE and Fragment A' after cleavage of TFM2 was confirmed by HPLC and LC-MS/MS analysis. In the case of BFM2 there was no Fragment A' formed as it does not have the TTR-binding module in the structure. The HPLC spectra are representatives of triplicate experiments (n=3).

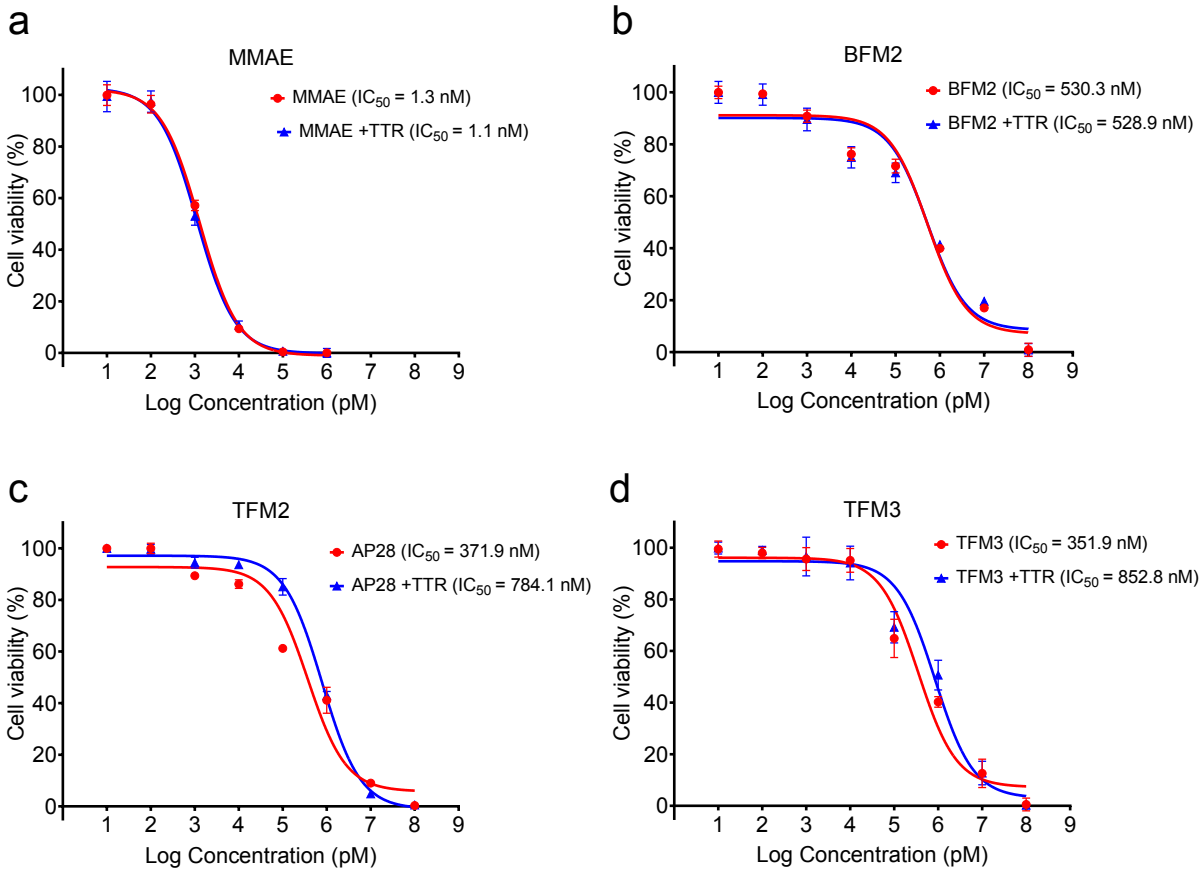


Figure S6. MTT cell proliferation assay was used to determine the cytotoxicity of MMAE, BFM2, TFM2 and TFM3 against HeLa cell line (PSMA- cells derived from cervical cancer cells) in the absence and presence of TTR.

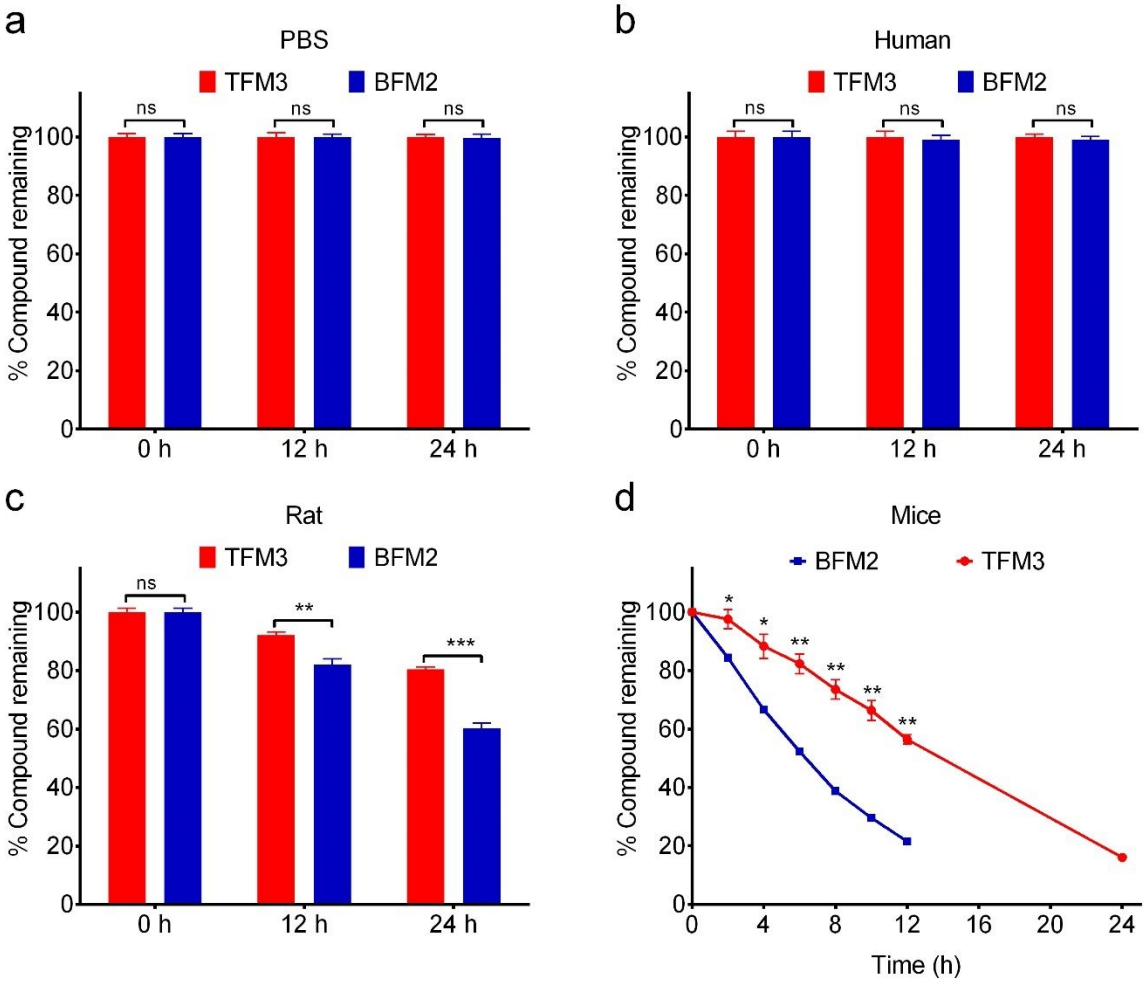


Figure S7. Evaluation of the stability of TFM3 and BFM2 in PBS buffer, human serum, rat serum, and mouse serum. Stability of TFM3 and BFM2 was evaluated in (a) PBS; (b) human serum; and (c) rat serum. Test compounds (50 μM) were incubated in PBS, human serum, or rat serum. The concentration of test compounds remaining in respective media were determined at 0, 12 and 24 h using HPLC. Bar graphs represent the mean of % compound remaining ± s.d. (n=3). (d) Stability of TFM3 and BFM2 in mouse serum was evaluated at different time points until 24 h. Test compounds (50 μM) were incubated in mouse serum. The concentration of test compounds remaining in respective medium were determined at different time points using HPLC. Each time point represents the mean of % compound remaining ± s.d. (n=3). The significance of the differences was measured by multiple t-test (unpaired, with Holm-sidak correction) (ns, not significant; *$p \leq 0.05$; **$p \leq 0.01$).

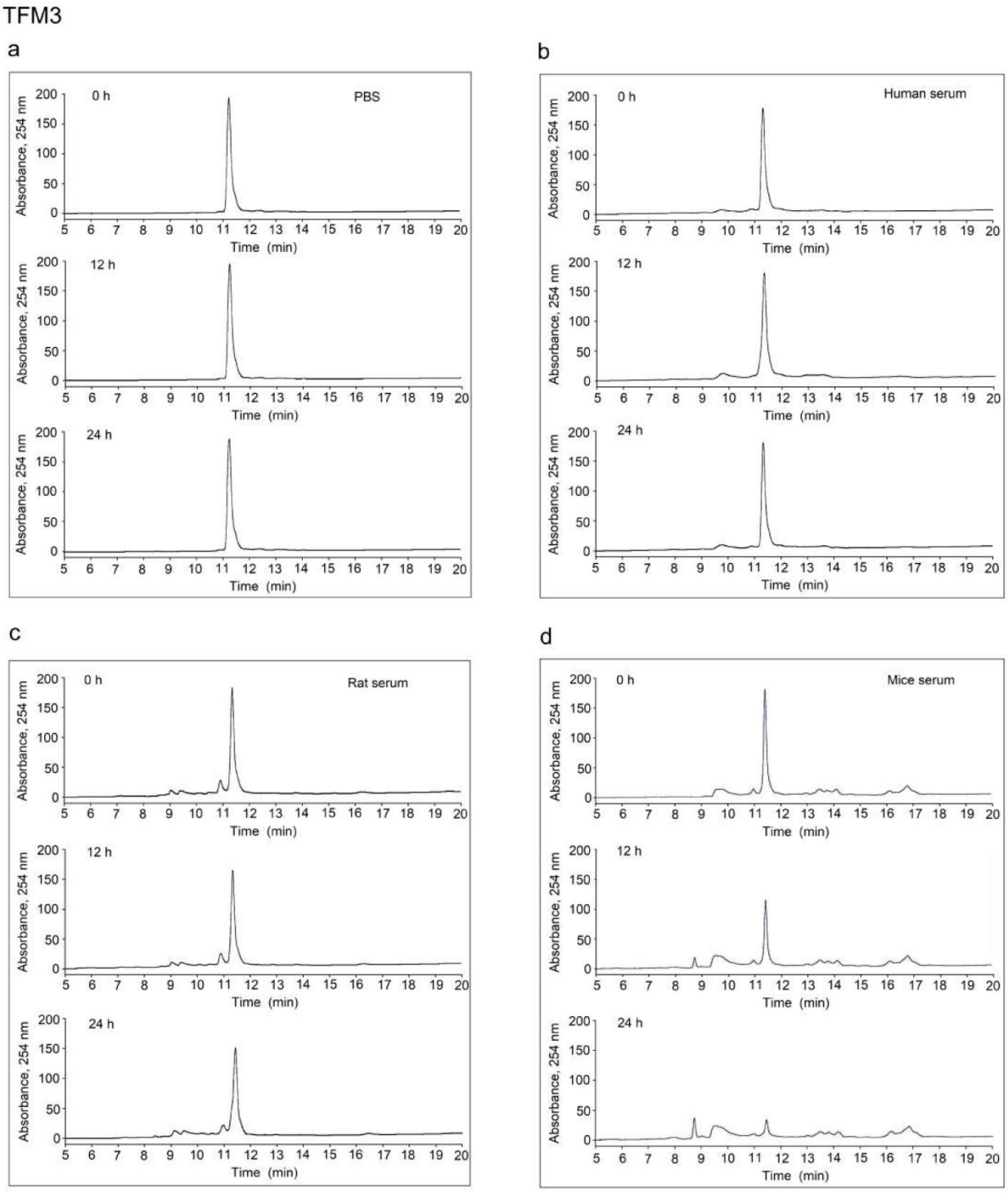


Figure S8. HPLC traces for the stability study of TFM3 in (a) PBS; (b) human serum; (c) rat serum; and (d) mice serum at 0 h, 12 h, and 24 h.

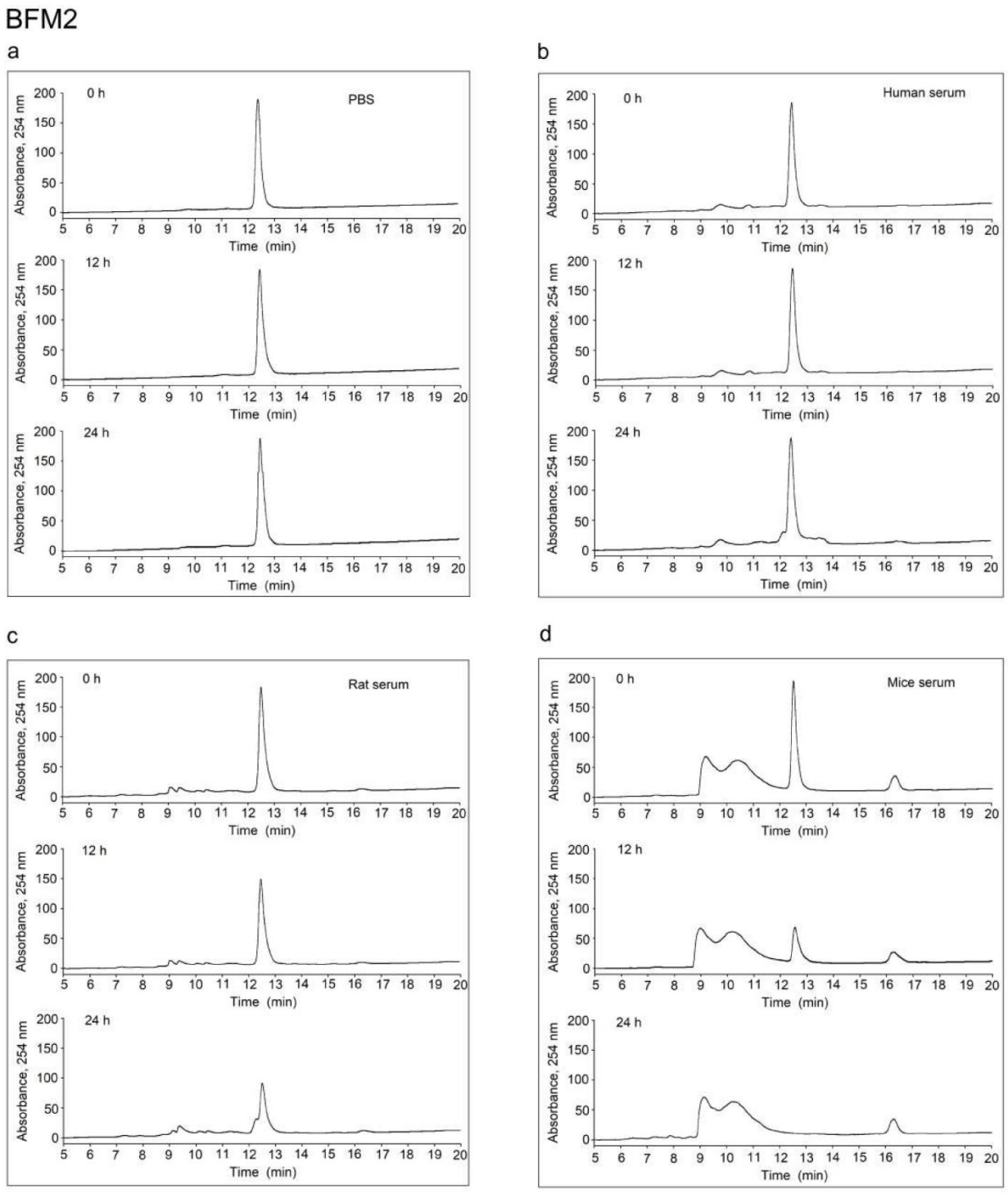


Figure S9. HPLC traces for the stability study of BFM2 in (a) PBS; (b) human serum; (c) rat serum; and (d) mice serum at 0 h, 12 h, and 24 h.

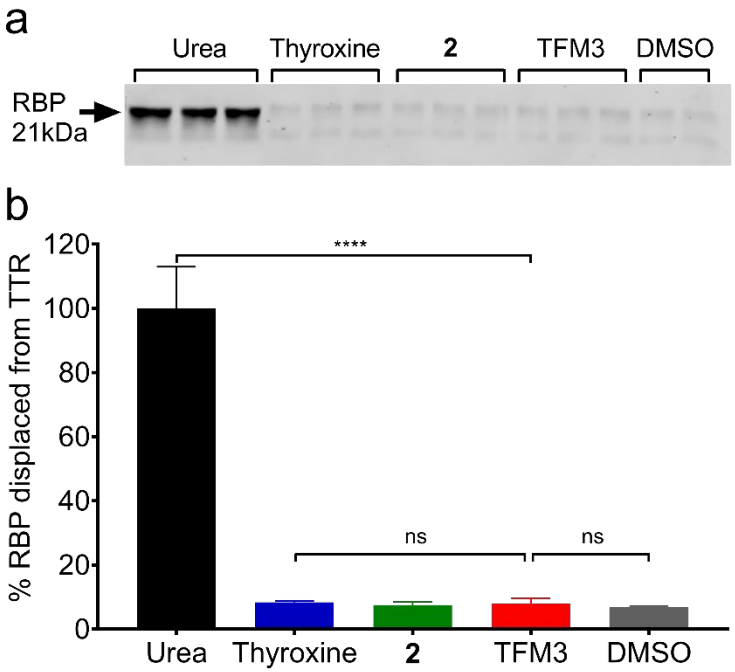


Figure S10. Binding of ligand **2** and TFM3 to TTR does not interfere with the holo-RBP binding to TTR. (a) Human serum (TTR concentration ~ 5 μM) was incubated with Thyroxine (T_4), ligand **2**, TFM3 (all compounds at 20μM final concentration) and DMSO in PBS buffer (pH 7) or with urea (8 M) buffer for 2 h at 37°C before cross-linking and immunoblotting. The membrane was incubated with rabbit anti-RBP antibody and then with IRdye800 donkey anti-rabbit secondary antibody. After incubation, the membrane was washed and scanned using LI-COR Odyssey® CLx Imaging System for quantification. The membrane image is a representation of replicate experiment (n=3). (b) Bar graph representing the mean (±s.d) (n=3) of %RBP displacement from TTR quantitated from three membranes. The significance of the differences was measured by one-way ANOVA followed by Tukey's multiple comparison test (ns, not significant; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$).

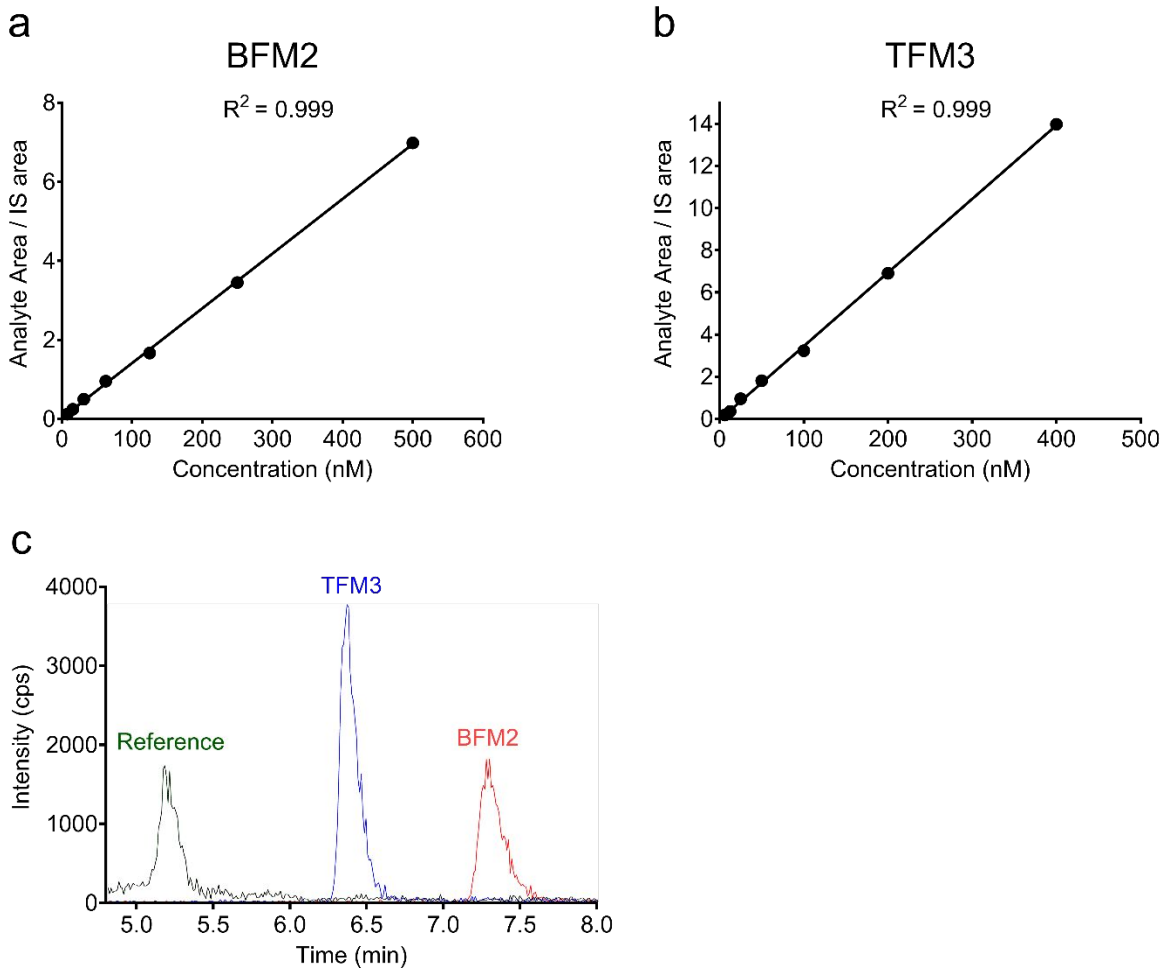


Figure S11. LC-MS/MS calibration curves used to quantitate (a) BFM2 and (b) TFM3 in rat plasma. (c) LC-MS/MS chromatogram of TFM3, BFM2, and internal standard (IS) reference in rat plasma. The identities of the compounds were determined using the following Q1/Q3 transition masses for TFM3 (1237.9/684.3), BFM2 (965.7/684.4), and IS reference (defluro-AG10) (273/92.9).

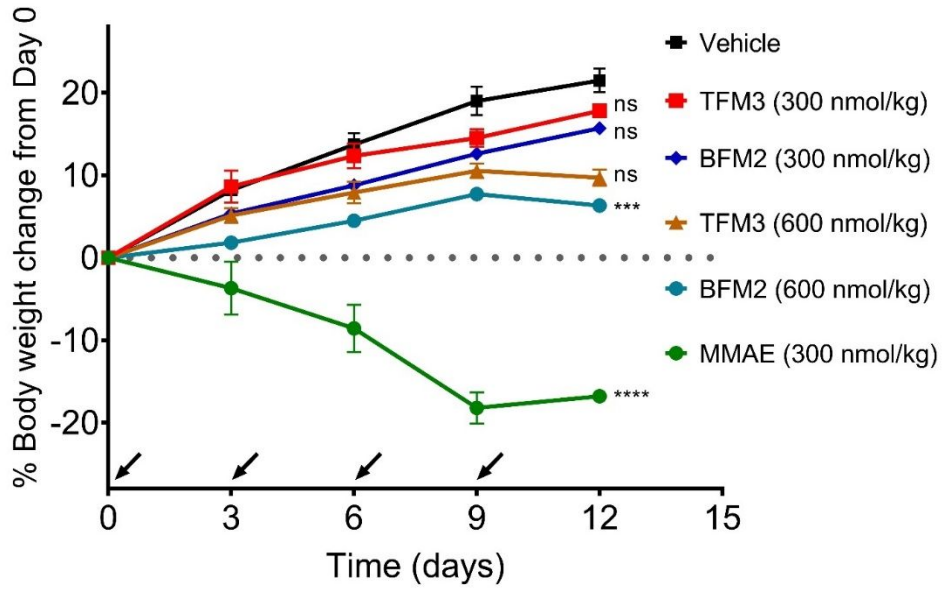
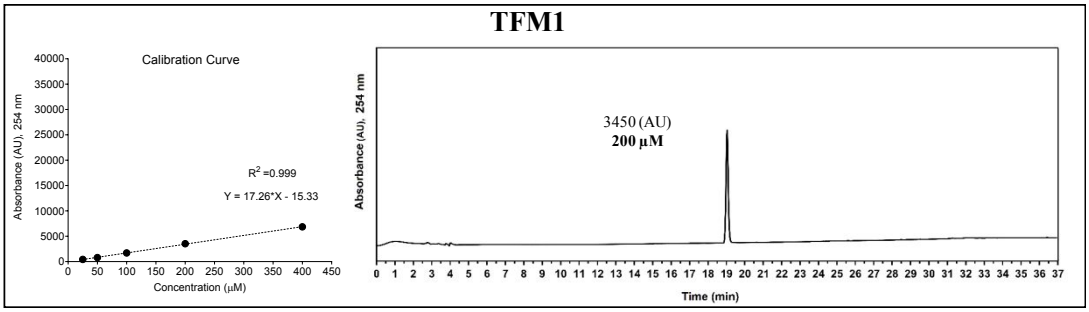
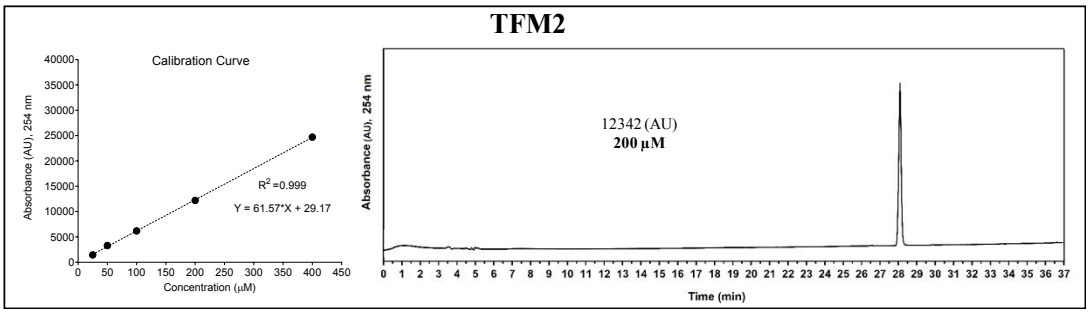


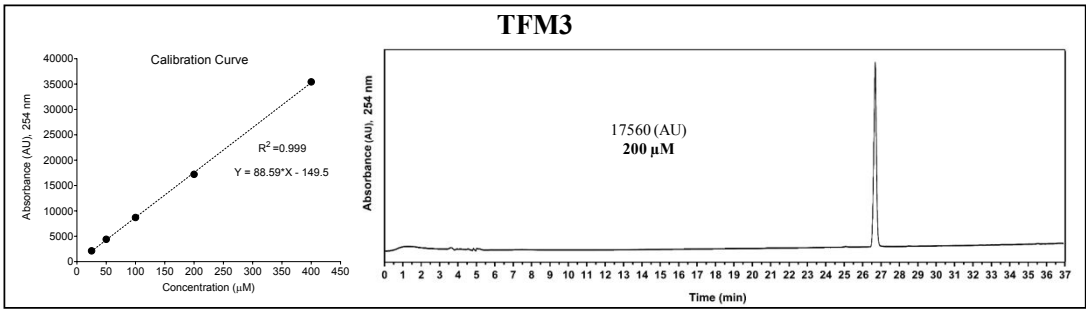
Figure S12. Preliminary evaluation of the toxicity of TFM3 and BFM2 in mice. Repeat dose toxicity of TFM3 and BFM2 were determined in CD-1 male mice model. Six groups of CD-1 male mice (n=4 per group) received a single dose of test compounds [BFM2 (300 nomol/kg or 600 nmol/kg), TFM3 (300 nmol/kg or 600 nmol/kg), MMAE (300 nmol/kg) or vehicle] every 3rd day for nine days (total four doses). The body weights of the animals were recorded every 3rd day. Each time point represents mean % body weight change from day 0 ± s.d. (n=4). The significance of the differences was measured by one-way ANOVA followed by Tukey's multiple comparison test (ns, not significant; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$).

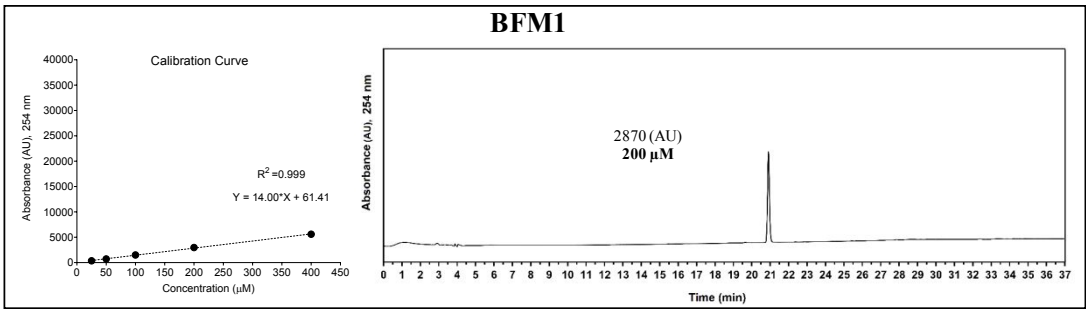
Additional Materials and Reagents. Human serum was purchased from Sigma (#H4522) [TTR concentration in serum was measured using nephelometric analyzer (28 mg/dL or 5 μM)]. Glutaraldehyde was purchased from Sigma (#G5882). RPMI-1640 Medium (HyClone, Utah, USA), fetal bovine serum (Gemini), penicillin/streptomycin (100 unit/ml and 100μg/ml; (Gibco, NY, USA) and L-Glutamine (Glutamax-100X, Gibco, NY, USA). CellBIND® 96 well clear plates (CLS3340-50EA) were purchased from Corning®. CellTiter 96 non-radioactive cell proliferation assay kit (cat# G4000, Promega, WI, USA) was used to perform the MTT (3-(4, 5-dimethylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide) assay for the test compounds in both the cell lines.

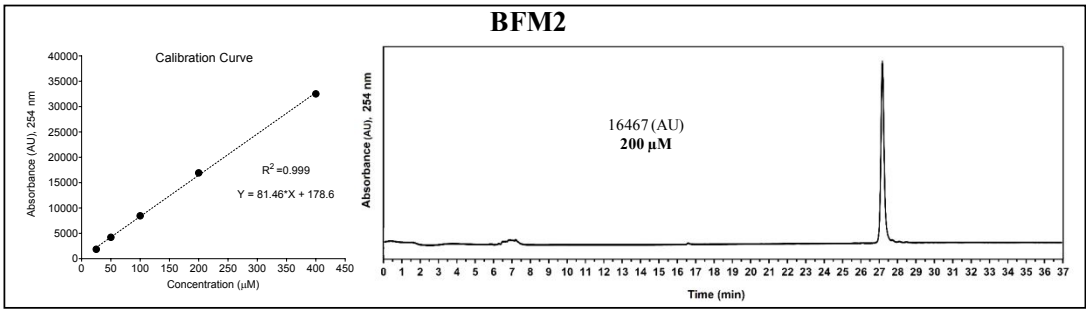
Evaluation of the Aqueous Solubility of Test Compounds. The aqueous solubility of BFMs and TFMs was evaluated (at 200 μM) in Phosphate buffer saline (PBS, pH 7.4) as reported earlier.[3] The samples were incubated at room temperature (25ºC) for 16 hours with moderate shaking (250 rpm). The samples were then centrifuged (2500 rpm for 3 min) and filtered. The concentration of test compounds in the filtrate was determined by HPLC. External standard from the same batch of test compounds was used to generate calibration curves. Each experiment was performed in duplicate. All BFMs and TFMs displayed excellent solubility (at least 200 μM) as shown below.











Aqueous solubility of TFMs and BFMs (tested at 200 μM) in PBS (pH 7.4)

HPLC Analysis of Serum or Plasma Samples. Analysis was performed on Agilent 1100 system or Waters Alliance e2695 system attached to Water 2998 PDA detector operating between the UV ranges of 200 nm and 800 nm and quantified using Chemstation and Empower 3 software, respectively. HPLC analysis was performed on a Waters XBridge C18 column (4.6 mm × 250 mm, 5 μm) and Waters Symmetry300 C4 column (2.1 mm × 150 mm, 5 μm) at ambient temperature upon injection of a 50 μl of each standard and/or sample to obtain the chromatogram.

Serum Stability Assay of TFM3 and BFM2. TFM3 and BFM2 (50 μM) was incubated in PBS, human serum, or rat serum at 37°C and samples (50 μl) were assayed at 0 h, 12 h, and 24 h time intervals. For the mouse serum, additional time points were included in the study. Samples were processed by adding 200 μl of solvent B (95% methanol and 0.1% Formic acid in water) followed by centrifuging at 15,000 rpm for 5 min (2x) and the supernatant was analyzed using HPLC. HPLC analysis was performed on a Waters Symmetry300 C4 column (2.1 mm × 150 mm, 5 μm) using a gradient method increasing linearly from 0-100% solvent B in 20 min. The mobile phase was composed of solvent A consisting of acetonitrile-water (5:95, v/v) containing 0.1% formic acid and solvent B consisting acetonitrile-water (95:5, v/v) containing 0.1% formic acid, at a flow rate of 0.5 ml/minute. The stability analysis of TFM3 and BFM2 (each at 5 μM) in mice serum was performed in the absence and presence of AG10 (10 μM) using the same procedure described above (using LC-MS/MS for analysis) to evaluate the protective effect of TTR on these molecules.

Chemical Synthesis and HPLC Purity Analysis

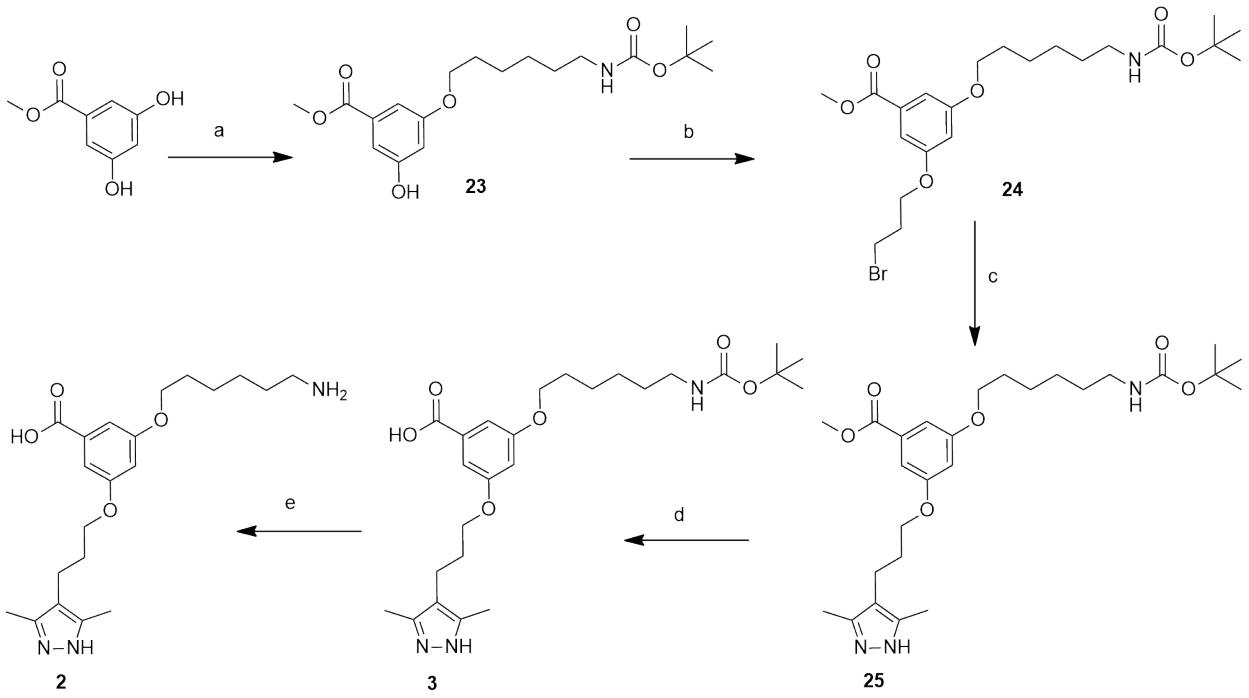
Materials for Chemical Synthesis. All reactions were carried out under an argon or nitrogen atmosphere using dry solvents under anhydrous conditions, unless otherwise noted. The solvents used were ACS grade from Fisher. Yields refer to chromatographically and spectroscopically (^{1}H NMR and ^{13}C NMR) homogeneous materials, unless otherwise noted. Reagents were purchased from Aldrich and Fisher, and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) carried out on EMD Millipore® silica gel 60 coated with fluorescent indicator F_{254} TLC plates (cat # 5737-7), using UV light and iodine chamber as visualizing agents. Normal phase flash column chromatography was carried out

using Combi Flash® Rf+ Lumen instrument (Teledyne ISCO) with High Performance Silica Flash Column (RediSep® Rf+ Gold), Preparative thin-layer chromatography (PTLC) separations were carried out on Analtech® 2mm (60F-254) (cat # 02015). ^{1}H NMR and ^{13}C NMR spectra were recorded on a Jeol JNM−ECA600 spectrometer and calibrated using residual undeuterated solvent as an internal reference. High-resolution mass spectra (HRMS) were determined by JEOL AccuTOF DART using Helium as an ionization gas and polyethylene glycol (PEG) as an external calibrating agent. Coupling constants (J) were expressed in Hertz.

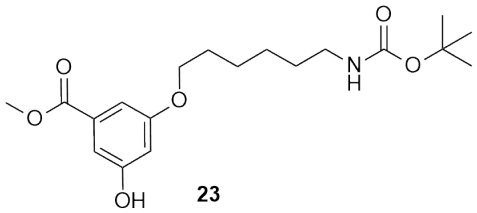
Preparative HPLC Method for Purification of TFMs and BFMs. The purification was performed on a Waters Delta 600 HPLC system connected to a photodiode array detector operating between the UV ranges of 210 – 600 nm, using Waters Masslynx V4.1 software. The HPLC analysis was performed on an XBridge™ Prep C18 Column (10 x 100 mm, 5 μM) at ambient temperature upon injection of 5 ml of each sample to obtain the chromatogram at 254 nm UV absorbance. The mobile phase was composed of solvent A consisting methanol-water (5:95, v/v) containing 0.1% formic acid and solvent B consisting methanol-water (95:5, v/v) containing 0.1% formic acid and delivered at a flow of 2.0 ml/min. The HPLC program was a gradient separation increasing linearly from 0-100 % solvent B.

Analytical HPLC Method for Evaluating the Purity of TFMs and BFMs. Detailed HPLC information of key compounds (traces, retention times, and %purity) are included below. The analysis of key compounds purity (>95% for all compounds) was performed using C18 and C4 reverse-phase HPLC columns on Agilent 1100 series HPLC system connected to a diode array detector operating between the UV ranges of 200 – 400 nm and quantified using Agilent Chemstation software. The HPLC analysis was performed on a Waters™ XBridge C18 column (4.6 X 150 mm, 5μm) or a Waters™ Symmetry300 C4 column (2.1 X 150 mm, 5 μm), eluting at 0.5 ml/min, at ambient temperature upon injection of 50 μl of each sample to obtain the chromatogram at 254nm UV absorbance. The mobile phase was composed of solvent A consisting methanol-water (5:95, v/v) containing 0.1% formic acid and solvent B consisting methanol-water (95:5, v/v) containing 0.1% formic acid. The HPLC program for the C18 column was a gradient method increasing linearly from 0-100 % solvent B at 10-20 minutes, followed by isocratic at 100% solvent B until 35 min, and then back to 0% of B at 37 min. For the C4 column, the method

was 0-100 % solvent B at 10-20 minutes, followed by isocratic at 100% solvent B until 28 min, and then back to 0% of B at 30 min.

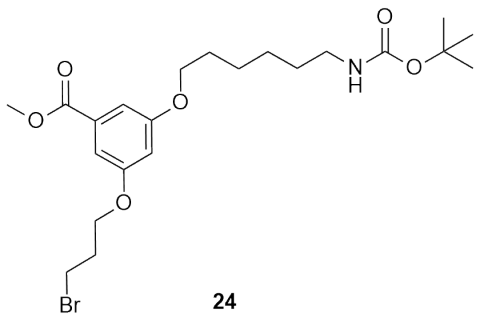


Scheme S1. Synthesis of TTR ligand **2**. a) tert-butyl (6-bromohexyl)carbamate, K_2CO_3, KI, MeCN, reflux, 16 h; b) 1,3- dibromopropane, K_2CO_3, DMF, rt, 16 h; c) i. acetylacetone, DBU, benzene, rt, 3 days; ii. hydrazine hydrate, ethanol, 90°C, 4 h; d) NaOH, MeOH/water, 50°C, 14 h; e) 20% TFA in DCM, rt, 3 h.



Methyl 3-((6-((tert-butoxycarbonyl)amino)hexyl)oxy)-5-hydroxybenzoate (23). To a solution of methyl 3,5- dihydroxybenzoate (0.77 g, 4.58 mmol, 1 equiv) and tert-butyl (6-bromohexyl)carbamate (1.15 g, 4.12 mmol, 0.9 equiv) in anhydrous MeCN (30 ml) was added K_2CO_3 (1.27 g, 9.16 mmol, 2 equiv) and KI (0.15 g, 0.92 mmol, 0.2 equiv). The suspension was heated to reflux for 16 h, filtered, and the solid was rinsed with MeCN. The filtrate was concentrated under reduced pressure. Water was added to the residue and the aqueous phase was

extracted with EtOAc (150 ml), washed with brine (3x50 ml) and dried with anhydrous sodium sulfate. The solution was filtered and concentrated and the residue was purified by flash column chromatography (silica gel, 1-10% EtOAc/hexanes) to afford compound **23** (1.06 g, 70% yield); ^{1}H NMR (CD_3OD, 600 MHz) δ 6.99-6.98 (m, 2H), 6.54 (t, 1H, J = 2.4 Hz), 3.96 (t, 2H, J = 6.6 Hz), 3.84 (s, 3H), 3.02 (t, 2H, J= 7.0 Hz), 1.77-1.73 (m, 2H), 1.50-1.35 (m, 6H), 1.40 (s, 9H); ^{13}C NMR (CD_3OD, 150 MHz): δ 25.48, 26.25, 27.44, 28.88, 29.57, 39.94, 51.27, 67.76, 78.47, 106.14, 106.50, 108.57, 131.71, 157.26, 158.53, 160.40, 167.20 ppm. HRMS (DART) *m/z:* calcd for $C_{19}H_{29}NO_6$+ H^+ 368.2068; found 368.2070 $(M + H)^+$.



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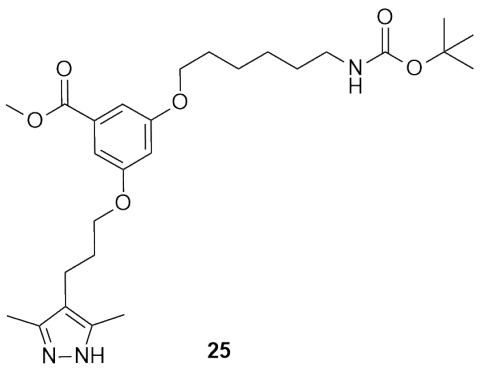
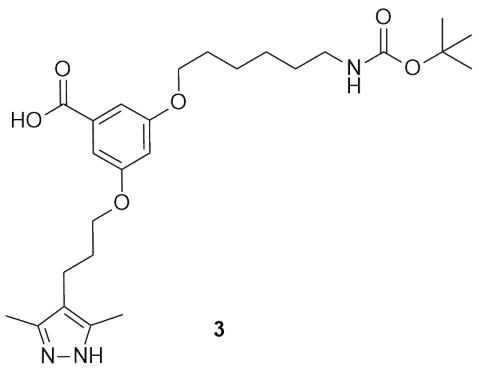
Methyl 3-(3-bromopropoxy)-5-((6-((tert-butoxycarbonyl)amino)hexyl)oxy)benzoate (24). To a solution of **23** (565.9 mg, 1.54 mmol, 1 equiv) and 1,3-dibromopropane (0.78 ml, 7.7 mmol, 5 equiv) in DMF (5 ml) was added K_2CO_3 (256 mg, 1.85 mmol, 1.2 equiv). The reaction mixture was stirred at room temperature for 16 h. The mixture was diluted with EtOAc (150 ml), washed with brine (3x50 ml) and dried with anhydrous sodium sulfate. The solution was filtered and concentrated. The residue was purified by flash column chromatography (silica gel, 1-10% EtOAc/hexanes) to afford compound **24** (646 mg, 86% yield); ^{1}H NMR (CD_3OD, 600 MHz) δ 7.12-7.11 (m, 2H), 6.69 (t, 1H, J = 2.4 Hz), 4.11 (t, 2H, J = 5.9 Hz), 3.97 (t, 2H, J = 6.4 Hz), 3.86 (d, J= 2.3 Hz, 3H), 3.60 (t, 2H, J= 6.5 Hz), 3.02 (t, 2H, J = 7.0 Hz), 2.30-2.26 (m, 2H), 1.79-1.74 (m, 2H), 1.50-1.35 (m, 6H), 1.40 (s, 9H); ^{13}C NMR (CD_3OD, 150 MHz): δ 25.47, 26.23, 27.45, 28.85, 29.13, 29.57, 32.15, 39.93, 51.41, 65.59, 67.93, 78.42, 106.03, 107.22, 107.65, 131.84, 157.26, 160.03, 160.42, 166.96 ppm. HRMS (DART) *m/z:* calcd for $C_{22}H_{34}BrNO_6$+ H^+ 488.1643; found 488.1629 $(M + H)^+$.



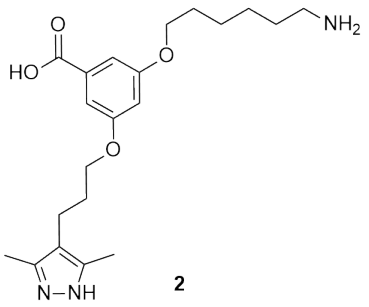
25

Methyl 3-((6-((tert-butoxycarbonyl)amino)hexyl)oxy)-5-(3-(3,5-dimethyl-1H-pyrazol-4-yl)propoxy)benzoate (25). A solution of **24** (618.74 mg, 1.27 mmol, 1 equiv) in benzene (5 ml) was added dropwise to a solution of acetyl acetone (0.26 ml, 2.54 mmol, 2 equiv) and DBU (0.38 ml, 2.54 mmol, 2 equiv) in benzene (7 ml). The reaction mixture was stirred at room temperature for 3 days. The mixture was filtered and passed through a pad of silica gel. The solvent was removed and the residue was dissolved in ethanol (5 ml). Hydrazine hydrate (0.17 ml, 3.18 mmol, 2.5 equiv) was added and the reaction was heated (90°C) under reflux for 4 h. The reaction was concentrated and purified by flash column chromatography (silica gel, 1-20% MeOH/CH_2Cl_2) to afford compound **25** (200 mg, 31% yield) in two steps; ^{1}H NMR (CD_3OD, 600 MHz) δ 7.10-7.08 (m, 2H), 6.65 (t, 1H, J = 2.4 Hz), 3.96 (t, 2H, J = 6.4 Hz), 3.89 (t, 2H, J = 6 Hz), 3.86 (s, 3H), 3.02 (t, 2H, J = 7.2 Hz), 2.55 (t, 2H, J = 7.2 Hz), 2.11 (s, 6H), 1.92-1.87 (m, 2H), 1.78-1.73 (m, 2H), 1.50-1.35 (m, 6H), 1.40 (s, 9H); ^{13}C NMR (CD_3OD, 150 MHz): δ 18.48, 25.48, 26.24, 27.45, 28.86, 29.42, 29.57, 39.93, 51.39, 66.53, 67.91, 78.46, 105.92, 107.26, 107.30, 113.86, 131.79, 157.25, 160.24, 160.41, 167.00 ppm. HRMS (DART) *m/z:* calcd for $C_{27}H_{41}N_3O_6$+ H^+ 504.3068; found 504.3054 $(M + H)^+$.



3

3-((6-((tert-butoxycarbonyl)amino)hexyl)oxy)-5-(3-(3,5-dimethyl-1H-pyrazol-4-yl)propoxy)benzoic acid (3). To a solution of **25** (115.76 mg, 0.23 mmol, 1 equiv) in a mixture of MeOH (4 ml) and water (1 ml) was added NaOH (18.4 mg, 0.46 mmol, 2 equiv). The reaction mixture was heated at 50°C for 14 h and then concentrated under reduced pressure. The residue was extracted with 50% MeOH/EtOAc. The combined organic extracts were concentrated under reduced pressure and the product was subject to purification by preparative HPLC to afford compound **3** (82.2 mg, 73% yield); (98% purity by HPLC): t_R (column) (C18) = 25.2 min; t_R (C4) = 21.6 min. ^{1}H NMR (CD_3OD, 600 MHz) δ 7.10-7.08 (m, 2H), 6.47 (t, 1H, J = 2.3 Hz), 3.95 (t, 2H, J = 6.4 Hz), 3.88 (t, 2H, J = 5.9 Hz), 3.02 (t, 2H, J= 7.0 Hz), 2.55 (t, 2H, J = 7.2 Hz), 2.11 (s, 6H), 1.91-1.86 (m, 2H), 1.73-1.77 (m, 2H), 1.50-1.35 (m, 6H), 1.40 (s, 9H); ^{13}C NMR (CD_3OD, 150 MHz): δ 18.50, 25.57, 26.31, 27.46, 29.00, 29.57, 39.99, 66.16, 67.65, 78.43, 103.58, 107.14, 107.17, 114.00, 139.98, 157.25, 159.71, 159.84, 173.74. HRMS (DART) *m/z:* calcd for $C_{26}H_{39}N_3O_6$+ H^+ 490.2912; found 490.2935 $(M + H)^+$.

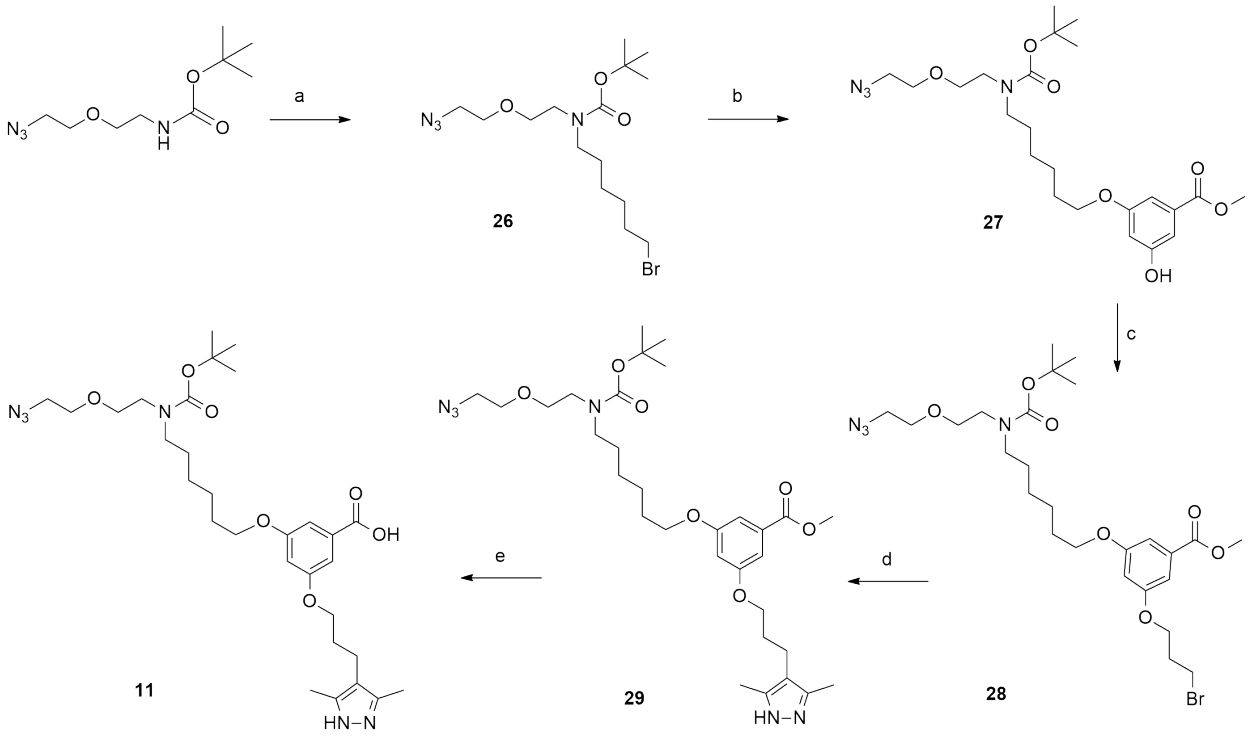


2

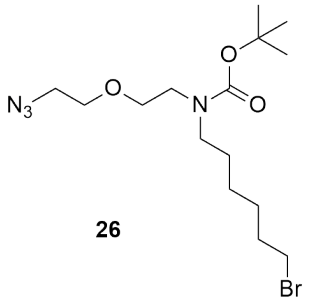
3-((6-Aminohexyl)oxy)-5-(3-(3,5-dimethyl-1H-pyrazol-4-yl)propoxy) benzoic acid (2). To a solution of **3** (60 mg, 0.123 mmol, 1 equiv) was added a mixture containing TFA and CH_2Cl_2, (1:4

ratio) (3 ml). The reaction was stirred at room temperature for 3 h. The solution was concentrated under reduced pressure and purified by preparative HPLC to afford compound **2** (41.65 mg, 87% yield); (98% purity by HPLC): t_R (column) (C18) = 21.6 min; t_R (C4) = 17.7 min. ^{1}H NMR (CD_3OD, 600 MHz) δ 7.10 (d, 2H, *J* = 2.4 Hz), 6.55 (t, 1H, *J* = 2.4), 3.97 (t, 2H, *J* = 6 Hz), 3.88 (t, 2H, *J* = 6 Hz), 2.91 (t, 2H, *J* = 7.2 Hz), 2.55 (t, 2H, *J* = 7.2 Hz), 2.11 (s, 6H), 1.91-1.87 (m, 2H), 1.79-1.75 (m, 2H), 1.68-1.63 (m, 2H), 1.54-1.49 (m, 2H), 1.47-1.41 (m, 2H); ^{13}C NMR (CD_3OD, 150 MHz): δ 9.23, 18.51, 25.31, 25.80, 27.18, 28.62, 29.50, 39.33, 48.52, 66.37, 67.57, 104.82, 107.34, 107.38, 113.94, 135.97, 141.96, 160.04, 170.73 ppm. HRMS (DART) *m/z:* calcd for $C_{21}H_{31}N_3O_4$+ H^+ 390.2388; found 390.2375 $(M + H)^+$.

Chemical Synthesis of 2PEG-modified ligand 2 (compound 11)

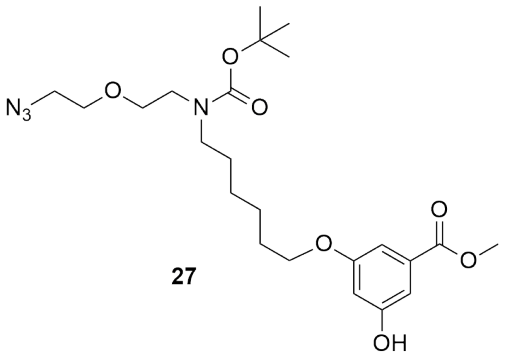


Scheme S2. Synthesis of 2PEG-modified ligand **2** (compound **11**). a) NaH, 1,6-dibromohexane, DMF, rt, 3 h; b) K_2CO_3, 3,5-dihydroxybenzoate, DMF, rt, 16 h; c) K_2CO_3,1,3-dibromopropane, DMF, rt, 16 h; d) i. acetylacetone, DBU, benzene, rt, 3 days; ii. hydrazine hydrate, ethanol, 90°C, 4 h; e) NaOH, MeOH/water, 50°C, 14 h.



26

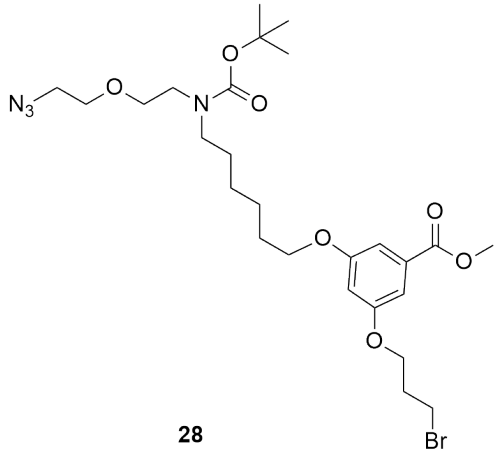
***tert*-Butyl (2-(2-azidoethoxy)ethyl)(6-bromohexyl)carbamate (26).** To a solution of *N*-[2-(2-azidoethoxy)ethyl]-,1,1-dimethylethyl ester (9 g, 39.11 mmol, 1 equiv) in anhydrous DMF (100 ml) was added NaH (3.13 g, 78.21 mmol, 2 equiv). After 15 min, 1, 6-dibromohexane (30.2 ml, 195.55 mmol, 5 equiv) was added dropwise to the solution. The reaction mixture was stirred at room temperature for 3 h. The mixture was quenched with water, diluted with EtOAc (600 ml), washed with brine (3x300 ml). The EtOAc fraction was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by flash column chromatography (silica gel, 1-50% EtOAc/hexanes) to afford compound **26** (11.076 g, 72% yield); ^{1}H NMR (CD_3OD, 600 MHz) δ 3.57 (t, 2H, *J* = 4.8 Hz), 3.54 (t, 2H, *J* = 5.4 Hz), 3.38 (t, 2H, *J* = 6.6 Hz), 3.34 (t, 2H, *J* = 5.4 Hz), 3.32-3.28 (m, 2H), 3.24-3.20 (m, 2H), 1.83-1.76 (m, 2H), 1.55-1.47 (m, 2H), 1.45-1.38 (m, 2H), 1.40 (s, 9H), 1.30-1.22 (m, 2H); ^{13}C NMR (CD_3OD, 150 MHz): δ 27.18, 28.95, 29.12, 29.57, 34.1, 34.5, 48.2, 48.4, 52.1, 71.28, 71.59, 81.14, 157.55 ppm. HRMS (DART) *m/z:* calcd for $C_{15}H_{29}BrN_4O_3 + H^+$ 393.1496*;* found 393.1500 $(M + H)^+$.



27

Methyl 3-((6-((2-(2-azidoethoxy)ethyl)(*tert*-butoxycarbonyl)amino)hexyl)oxy)-5-hydroxybenzoate (27). To a solution of **26** (11.0 g, 27.97 mmol, 1 equiv) and methyl 3,5-dihydroxybenzoate (14.12 g, 83.90 mmol, 3 equiv) in anhydrous DFM (100 ml) was added K_2CO_3

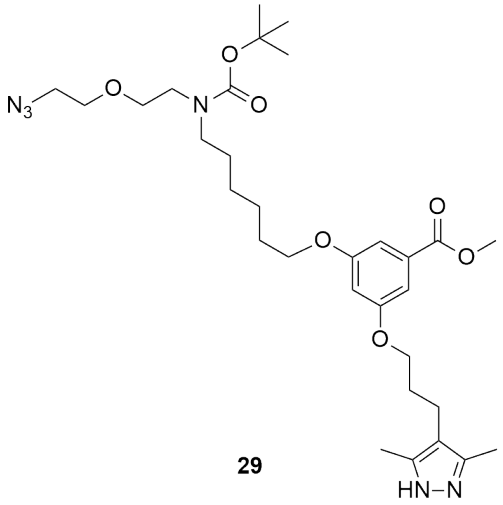
(5.80 g, 41.96 mmol, 1.5 equiv). The suspension was stirred at room temperature for 16 h. The suspension was quenched with water, diluted with EtOAc (500 ml), washed with brine (3x300 ml). The EtOAc fraction was dried over anhydrous sodium sulfate and concentrated in vacuo. The solution was concentrated under reduced pressure and the residue was purified by flash column chromatography (silica gel, 1-60% EtOAc/hexanes) to afford compound **27 (**5.91 g, 44% yield); ^{1}H NMR (CD_3OD, 600 MHz) δ 6.95-6.93 (m, 2H), 6.49 (t, 1H, *J* = 2.4 Hz), 3.89 (t, 2H, *J* = 6.6 Hz), 3.79 (s, 3H), 3.55 (t, 2H, *J* = 4.8 Hz), 3.52 (t, 2H, *J* = 5.4 Hz), 3.32 (t, 2H, *J* = 5.4 Hz), 3.30-3.26 (m, 2H), 3.24-3.19 (m, 2H), 1.74-1.67 (m, 2H), 1.56-1.48 (m, 2H), 1.47-1.41 (m, 2H), 1.38 (s, 9H), 1.32-1.25 (m, 2H); ^{13}C NMR (CD_3OD, 150 MHz): δ 27.07, 27.74, 28.94, 29.67, 30.41, 48.20, 48.44, 52.06, 52.82, 69.26, 70.62, 71.25, 81.16, 107.71, 107.92, 110.12, 133.26, 157.72, 160.07, 161.92, 168.72 ppm. HRMS (DART) *m/z:* calcd for $C_{23}H_{36}N_4O_7 + H^+$ 481.2657; found 481.2688 $(M + H)^+$.



28

Methyl 3-((6-((2-(2-azidoethoxy)ethyl)(*tert*-butoxycarbonyl)amino)hexyl)oxy)-5-(3-bromopropoxy)benzoate (28). To a solution of **27** (5.00 g, 10.4 mmol, 1 equiv) and 1,3-dibromopropane (5.28 ml, 52 mmol, 5 equiv) in DMF (60 ml) was added K_2CO_3 (2.153 g, 15.6 mmol, 1.5 equiv). The suspension was stirred at room temperature overnight. The reaction was quenched with water, diluted with EtOAc (200 ml), and washed with brine (3x100 ml). The EtOAc fraction was dried over anhydrous sodium sulfate and concentrated in vacuo. The solution was concentrated under reduced pressure and the residue was purified by flash column chromatography (silica gel, 1-50% EtOAc/hexanes) to afford compound **28 (**5.43 g, 87% yield); ^{1}H NMR (CD_3OD, 600 MHz) δ 7.07 (d, 2H, *J* = 1.8 Hz), 6.65 (t, 1H, *J* = 2.4 Hz), 4.06 (t, 2H, *J* = 6 Hz), 3.93 (t, 2H,

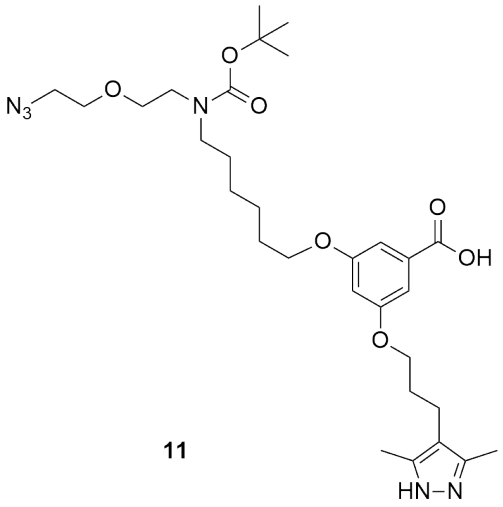
J = 6 Hz), 3.81 (s, 3H), 3.58-3.54 (m, 4H), 3.52 (t, 2H, J = 5.4 Hz), 3.32 (t, 2H, J = 5.4 Hz), 3.30-3.26 (m, 2H), 3.24-3.19 (m, 2H), 2.25- 2.20 (m, 2H), 1.76-1.68 (m, 2H), 1.57-1.49 (m, 2H), 1.48-1.42 (m, 2H), 1.38 (s, 9H), 1.32-1.25 (m, 2H); ^{13}C NMR (CD_3OD, 150 MHz): δ 27.07, 27.73, 28.94, 29.68, 30.38, 30.68, 33.69, 49.73, 49.77, 52.07, 52.94, 67.13, 69.44, 70.76, 71.31, 81.16, 107.57, 108.78, 109.21, 133.39, 157.72, 161.59, 161.91, 168.45. HRMS (DART) *m/z:* calcd for $C_{26}H_{41}BrN_4O_7$ + H^+ 601.2232; found 601.2242 $(M + H)^+$.



29

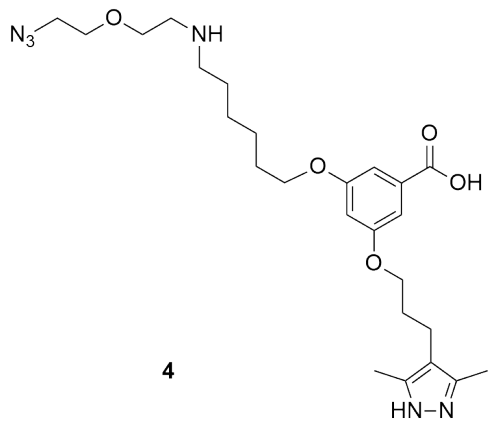
Methyl 3-((6-((2-(2-azidoethoxy)ethyl)(*tert*-butoxycarbonyl)amino)hexyl)oxy)-5-(3-(3,5-dimethyl-1*H*-pyrazol-4-yl)propoxy)benzoate (29). To a solution of **28** (3.50 g, 5.82 mmol, 1 equiv), in benzene (15 ml) was added dropwise a solution of acetyl acetone (1.195 ml, 11.64 mmol, 2 equiv) and DBU (1.74 ml, 11.64 mmol, 2 equiv) in benzene (25 ml). The reaction mixture was stirred at room temperature for 3 days. The mixture was filtered and passed through a pad of silica gel. The solvent was removed and the residue was dissolved in anhydrous ethanol (30 ml). Hydrazine hydrate (0.76 ml, 14.55 mmol, 2.5 equiv) was added and the reaction was heated under reflux for 4 h. The reaction was concentrated and purified by flash column chromatography (silica gel, 1-100% EtOAc/hexanes) to afford compound **29** (1.29 g, 36% yield); ^{1}H NMR (CD_3OD, 600 MHz) δ 7.05-7.02 (m, 2H), 6.60 (t, 1H, J = 2.4 Hz), 3.90 (t, 2H, J = 6 Hz), 3.84 (t, 2H, J = 6 Hz), 3.81 (s, 3H), 3.56-3.52 (m, 2H), 3.51 (t, 2H, J = 5.4 Hz), 3.31 (t, 2H, J = 5.4 Hz), 3.28-3.25 (m, 2H), 3.23-3.19 (m, 2H), 2.5 (t, 2H, J = 7.2 Hz), 2.06 (s, 6H), 1.87-1.81 (m, 2H), 1.74-1.68 (m, 2H), 1.55-1.48 (m, 2H), 1.47-1.40 (m, 2H), 1.38 (s, 9H), 1.31-1.24 (m, 2H); ^{13}C NMR (CD_3OD, 150 MHz): δ 19.89, 26.94, 27.61, 28.81, 29.54, 30.25, 30.84, 48.10, 48.30, 51.92, 52.79, 67.93, 69.27,

70.81, 71.15, 81.00, 107.32, 108.67,108.71, 115.26, 133.19, 143.48, 157.43, 161.63, 161.78, 168.36. HRMS (DART) *m/z:* calcd for $C_{31}H_{48}N_6O_7$ + H^+ 617.3658; found 617.3662 $(M + H)^+$



11

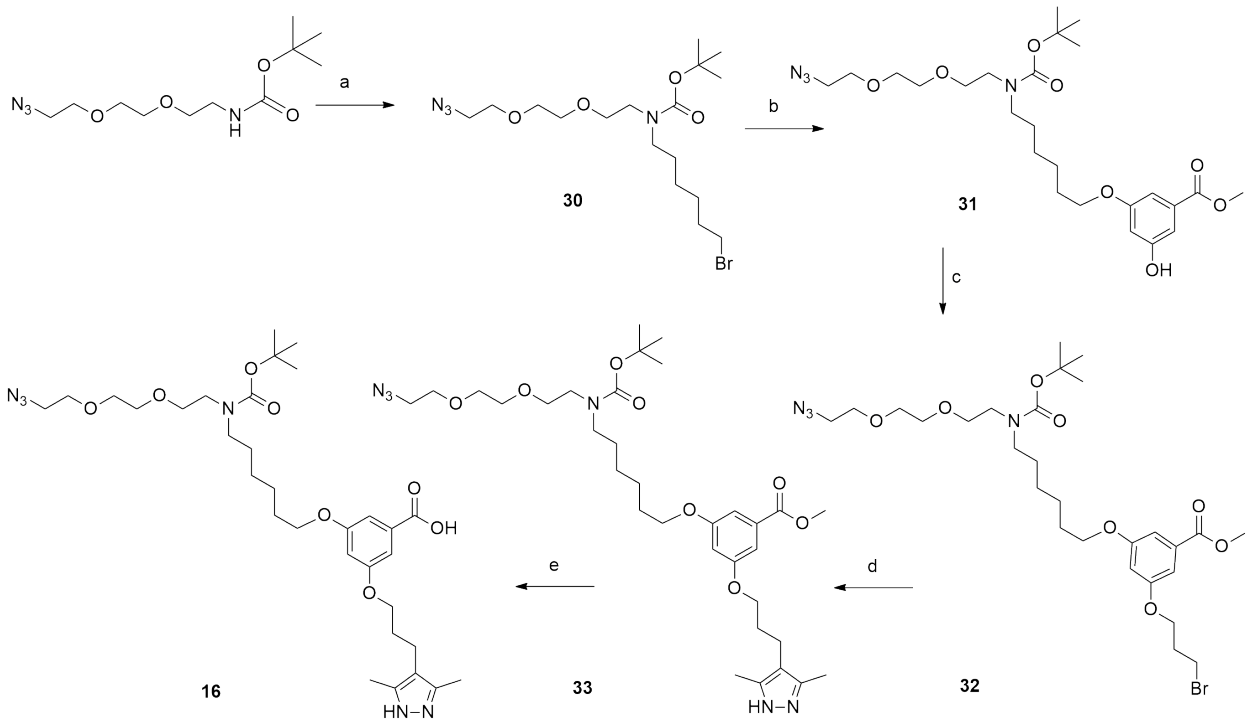
3-((6-((2-(2-Azidoethoxy)ethyl)(*tert*-butoxycarbonyl)amino)hexyl)oxy)-5-(3-(3,5-dimethyl-1*H*-pyrazol-4-yl)propoxy)benzoic acid (11). To a solution of **29** (1.20 g, 1.95 mmol, 1 equiv) in a mixture of MeOH (8 ml) and water (2 ml) was added NaOH (0.156 g, 3.9 mmol, 2 equiv). The reaction mixture was heated at 50°C for 14 h and then concentrated under reduced pressure. The residue was extracted with 50% MeOH/EtOAc. The combined organic extracts were concentrated under reduced pressure and the product was subject to purification by preparative HPLC to afford compound **11** (0.928 g, 79% yield); ^{1}H NMR (CD_3OD, 600 MHz) δ 7.04-7.01 (m, 2H), 6.56 (t, 1H, *J* = 2.4 Hz), 3.88 (t, 2H, *J* = 6 Hz), 3.82 (t, 2H, *J* = 6 Hz), 3.53-3.5 (m, 2H), 3.49(t, 2H, *J* = 5.4 Hz), 3.29 (t, 2H, *J* = 5.4 Hz), 3.26-3.22 (m, 2H), 3.18(t, 2H, *J* = 7.2), 2.48 (t, 2H, *J* = 7.2 Hz), 2.04 (s, 6H), 1.85-1.79 (m, 2H), 1.72-1.65 (m, 2H), 1.52-1.45 (m, 2H), 1.46-1.38 (m, 2H), 1.35 (s, 9H), 1.28-1.23 (m, 2H); ^{13}C NMR (CD_3OD, 150 MHz): δ 10.75, 20.03, 27.08, 27.74, 28.95, 29.68, 30.41, 30.98, 48.22, 48.43, 51.06, 68.02, 69.36, 70.77, 71.18, 81.16, 107.29, 109.03,109.71, 115.50, 134.16, 143.50, 157.59, 161.70, 161.86, 169.9. HRMS (DART) *m/z:* calcd for $C_{30}H_{46}N_6O_7$ + H^+ 603.3501; found 603.3510 $(M + H)^+$.



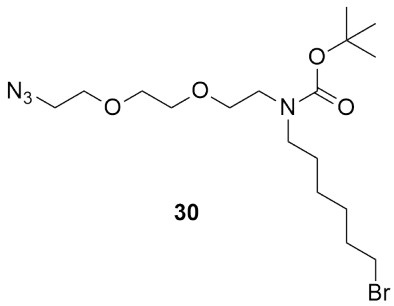
4

3-((6-((2-(2-azidoethoxy)ethyl)amino)hexyl)oxy)-5-(3-(3,5-dimethyl-1H-pyrazol-4-yl)propoxy)benzoic acid (4). To a solution of **11** (200 mg, 0.33 mmol, 1 equiv) was added a mixture containing TFA and CH_2Cl_2, (1:4 ratio) (5 mL). The reaction was stirred at room temperature for 2 h. The solution was concentrated under reduced pressure and purified by preparative HPLC to afford compound **4** (154 mg, 93% yield); (98% purity by HPLC): t_R (column) (C18) = 22.2 min; t_R (C4) = 18.2 min. ^{1}H NMR (CD_3OD, 600 MHz) 7.02 (d, 2H, J = 2.4 Hz), 6.47 (t, 1H, J = 2.4), 3.89 (t, 2H, J = 6 Hz), 3.81 (t, 2H, J = 6 Hz), 3.69-3.65 (m, 2H), 3.61-3.58 (m, 2H), 3.37-3.34 (m, 2H), 3.15-3.10 (m, 2H), 2.96-2.92 (m, 2H), 2.48 (t, 2H, J = 7.2 Hz), 2.04 (s, 6H), 1.84-1.78 (m, 2H), 1.72-1.66 (m, 2H), 1.66-1.59 (m, 2H), 1.47-1.41 (m, 2H), 1.40-1.33 (m, 2H); ^{13}C NMR (CD_3OD, 150 MHz): δ 10.76, 20.03, 26.84, 27.26, 27.41, 30.12, 31.05, 48.91, 51.82, 67.16, 67.88, 69.06, 71.28, 71.65, 106.17, 108.82, 108.90, 115.45, 138.10, 143.48, 161.51, 169.37. HRMS (DART) m/z: calcd for $C_{25}H_{38}N_6O_5 + H^+$ 503.2976; found 503.3004 $(M + H)^+$.

Chemical Synthesis of 3PEG-modified ligand 2 (compound 16)

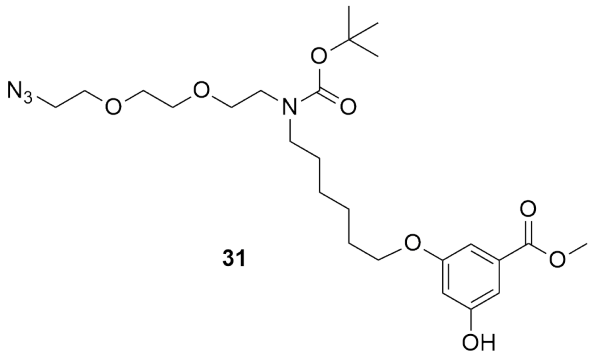


Scheme S3. Synthesis of 3PEG-modified ligand **2** (compound **16**). a) NaH, 1,6-dibromohexane, DMF, rt, 3 h; b) K_2CO_3, 3,5-dihydroxybenzoate, DMF, rt, 16 h; c) K_2CO_3,1,3-dibromopropane, DMF, rt, 16 h; d) i. acetylacetone, DBU, benzene, rt, 3 days; ii. hydrazine hydrate, ethanol, 90^{o}C, 4 h; e) NaOH, MeOH/water, 50^{o}C, 14 h.



30

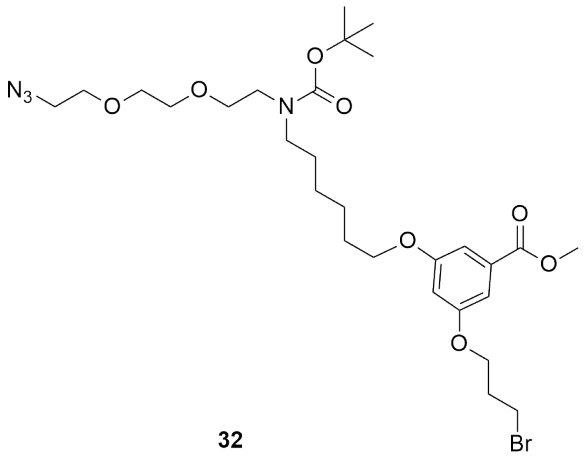
***tert*-Butyl (2-(2-(2-azidoethoxy)ethoxy)ethyl)(6-bromohexyl)carbamate (30).** To a solution of *tert*-Butyl 2-(2-azidoethoxy)ethoxyethylcarbamate (3.88 g, 14.14 mmol, 1 equiv) in anhydrous DMF (80 ml) was added NaH (1.13 g, 28.29 mmol, 2 equiv). After 15 min, 1, 6-dibromohexane (11 ml, 71.24 mmol, 5.04 equiv) was added dropwise to the solution. The reaction mixture was stirred at room temperature for 3 h. The mixture was quenched with water, diluted with EtOAc (600 ml), washed with brine (3x300 ml). The EtOAc fraction was dried over anhydrous sodium sulfate and concentrated in vacuo. The residue was purified by flash column chromatography (silica gel, 1-50% EtOAc/hexanes) to afford compound **30** (4.690 g, 76% yield); ^{1}H NMR (CD_3OD, 600 MHz) δ 3.61-3.59 (m, 2H), 3.58-3.53 (m, 4H,), 3.52 (t, 2H, *J* = 6Hz), 3.38 (t, 2H, *J* = 6.6 Hz), 3.32-3.28 (m, 4H), 3.19 (t, 2H, *J* = 6.6 Hz), 1.82-1.75 (m, 2H), 1.54-1.45 (m, 2H), 1.44-1.38 (m, 2H), 1.38 (s, 9H), 1.28-1.21 (m, 2H); ^{13}C NMR (CD_3OD, 150 MHz): δ 27.19, 28.95, 29.13, 29.6, 34.12, 34.5, 48.16, 48.32, 51.99, 71.92, 71.40, 71.82, 81.12, 157.55 ppm. HRMS (DART) *m/z:* calcd for $C_{17}H_{33}BrN_4O_4$ + H^+ 437.1758; found 437.1762 $(M + H)^+$.



31

Methyl 3-((6-((2-(2-(2-azidoethoxy)ethoxy)ethyl)(*tert*-butoxycarbonyl)amino)hexyl)oxy)-5-hydroxybenzoate (31). To a solution of **30** (4.56 g, 10.45 mmol, 1 equiv) and methyl 3,5-dihydroxybenzoate (5.27 g, 31.34 mmol, 3 equiv) in anhydrous DFM (80 ml) was added K_2CO_3

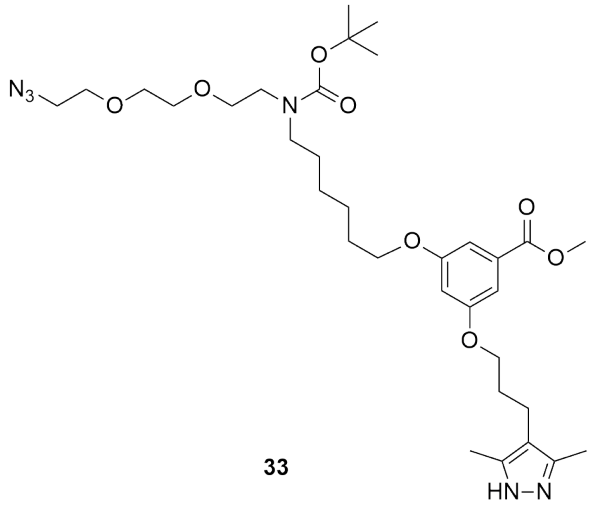
(2.16 mg, 15.63 mmol, 1.5 equiv). The suspension was stirred at room temperature for 16 h. The suspension was quenched with water, diluted with EtOAc (500 ml), washed with brine (3x300 ml). The EtOAc fraction was dried over anhydrous sodium sulfate and concentrated in vacuo. The solution was concentrated under reduced pressure and the residue was purified by flash column chromatography (silica gel, 1-5% MeOH/CH_2Cl_2) to afford compound **31** (1.91 g, 35% yield); ^{1}H NMR (CD_3OD, 600 MHz) δ 6.96-6.93 (m, 2H), 6.49 (t, 1H, *J* = 2.4 Hz), 3.89 (t, 2H, *J* = 6.6 Hz), 3.79 (s, 3H), 3.59-3.57 (m, 2H), 3.57-3.53 (m, 4H), 3.52 (t, 2H, *J* = 6 Hz), 3.31 (t, 2H, *J* = 6 Hz), 3.29-3.26 (m, 2H), 3.23-3.18 (m, 2H), 1.74-1.68 (m, 2H), 1.56-1.48 (m, 2H), 1.47-1.41 (m, 2H), 1.38 (s, 9H), 1.32-1.25 (m, 2H); ^{13}C NMR (CD_3OD, 150 MHz): δ 27.09, 27.74, 28.94, 29.46, 30.41, 48.12, 48.32, 51.97, 52.82, 69.27, 70.92, 71.39,71.82, 81.13, 107.70, 107.92, 110.12, 133.27, 157.62, 160.08, 161.92, 168.71 ppm. HRMS (DART) *m/z:* calcd for $C_{25}H_{40}N_4O_8$ + H^+ 525.2919*;* found 525.2920 (M + H^+).



32

Methyl 3-((6-((2-(2-(2-azidoethoxy)ethoxy)ethyl)(*tert*-butoxycarbonyl)amino)hexyl)oxy)-5-(3-bromopropoxy)benzoate (32). To a solution of **31** (1.5 g, 2.87 mmol, 1 equiv) and 1,3-dibromopropane (1.45ml, 14.3 mmol, 5 equiv) in DMF (20 ml) was added K_2CO_3 (0.594 g, 4.3 mmol, 1.5 equiv). The suspension was stirred at room temperature for 16 h. The reaction was quenched with water, diluted with EtOAc (300 ml), and washed with brine (3x200 ml). The EtOAc fraction was dried over anhydrous sodium sulfate and concentrated in vacuo. The solution was concentrated under reduced pressure and the residue was purified by flash column chromatography (silica gel, 1-50% EtOAc/hexanes) to afford compound **32** (1.57 g, 85% yield)**;** ^{1}H NMR (CD_3OD, 600 MHz) δ 7.07 (d, 2H, *J* = 2.4 Hz), 6.65 (t, 1H, *J* = 2.4 Hz), 4.06 (t, 2H, *J* = 6 Hz), 3.93 (t, 2H,

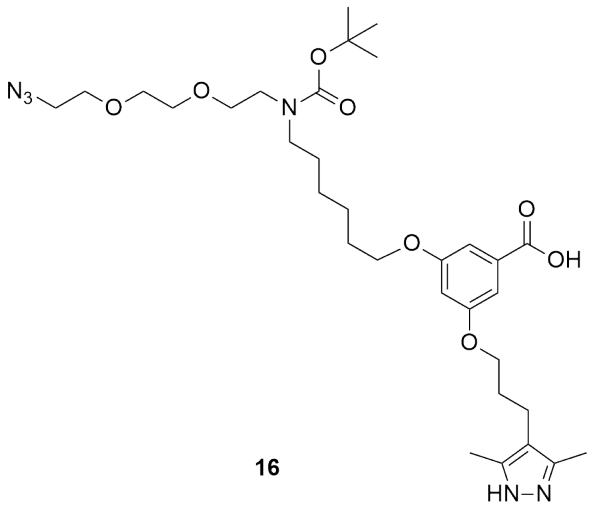
J = 6 Hz), 3.82 (s, 3H), 3.60-3.57 (m, 2H), 3.56-3.54 (m, 6H), 3.52 (t, 2H, J = 6 Hz), 3.31 (t, 2H, J = 5.4 Hz), 3.28 (t, 2H, J = 4.8 Hz), 3.23-3.18 (m, 2H), 2.26- 2.20 (m, 2H), 1.76-1.69 (m, 2H), 1.58-1.49 (m, 2H), 1.48-1.41 (m, 2H), 1.38 (s, 9H), 1.32-1.26 (m, 2H); ^{13}C NMR (CD_3OD, 150 MHz): δ 27.08, 27.74, 28.96, 29.70, 30.39, 30.71, 33.68, 49.14, 48.32, 51.97, 52.95, 67.11, 69.43, 70.94, 71.39,71.81, 81.11, 107.55, 108.77, 109.21, 133.37, 157.67, 161.56, 161.91, 168.43. HRMS (DART) *m/z*: calcd for $C_{28}H_{45}BrN_4O_8$ + H^+ 645.2494; found 645.2493 $(M + H)^+$.



33

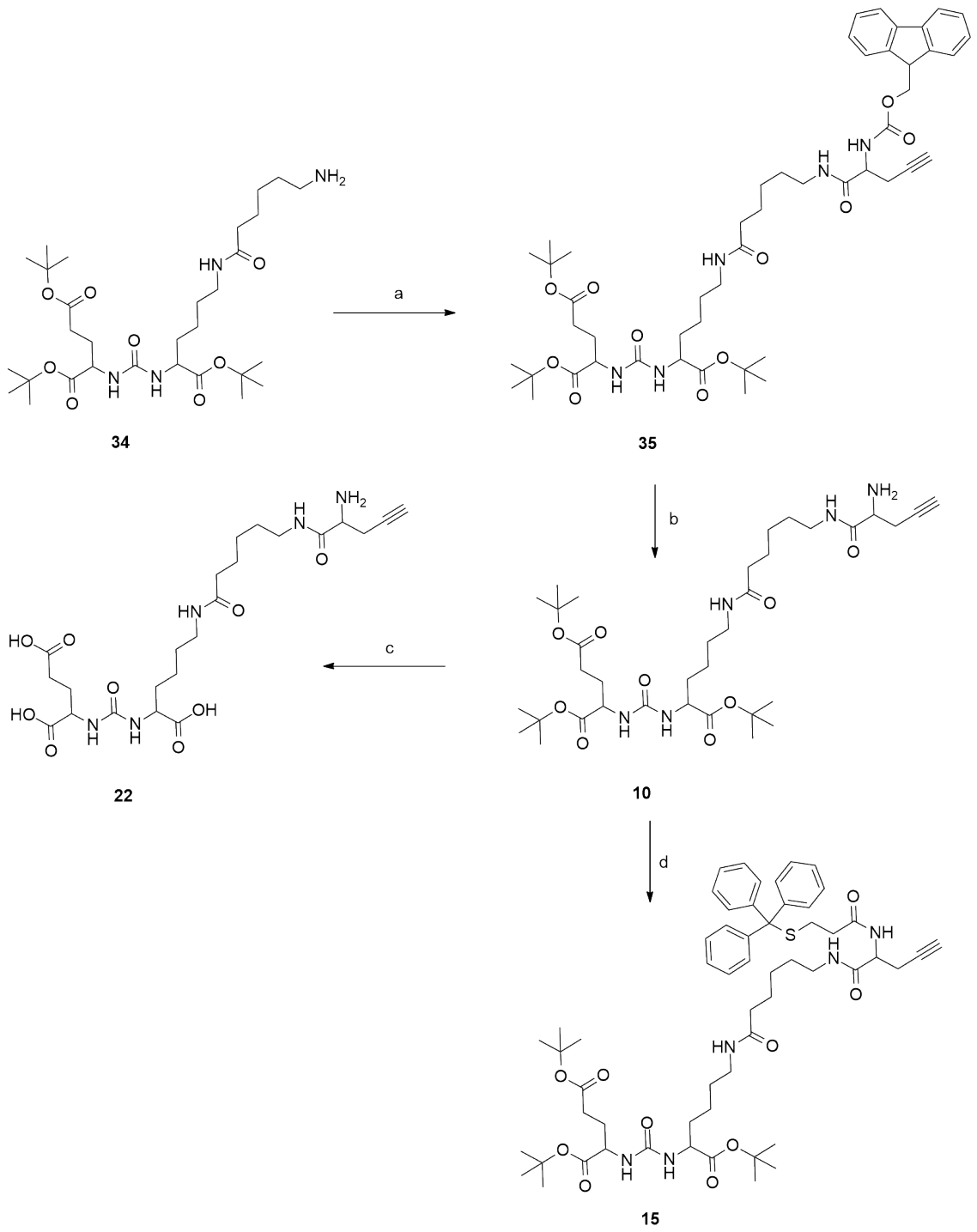
Methyl 3-((6-((2-(2-(2-azidoethoxy)ethoxy)ethyl)(*tert*-butoxycarbonyl)amino)hexyl)oxy)-5-(3-(3,5-dimethyl-1H-pyrazol-4-yl)propoxy)benzoate (33). To a solution of **32** (1.22 g, 1.89 mmol, 1 equiv), in benzene (7 ml) was added dropwise a solution of acetyl acetone (0.388 ml, 3.78 mmol, 2 equiv) and DBU (0.564 ml, 3.78 mmol, 2 equiv) in benzene (13 ml). The reaction mixture was stirred at room temperature for 3 days. The mixture was filtered and passed through a pad of silica gel. The solvent was removed and the residue was dissolved in anhydrous ethanol (10 ml). Hydrazine hydrate (0.232 ml, 4.73 mmol, 2.5 equiv) was added and the reaction was heated under reflux for 4 h. The reaction was concentrated and purified by flash column chromatography (silica gel, 1-20% MeOH/CH_2Cl_2) to afford compound **33** (413 mg, 33% yield); ^{1}H NMR (CD_3OD, 600 MHz) δ 7.05-7.02 (m, 2H), 6.60 (t, 1H, J = 2.4 Hz), 3.91 (t, 2H, J = 6 Hz), 3.84 (t, 2H, J = 6 Hz), 3.81 (s, 3H), 3.59-3.57 (m, 2H), 3.56-3.53 (m, 4H), 3.51 (t, 2H, J = 6 Hz), 3.30 (t, 2H, J = 5.4 Hz), 3.27 (t, 2H, J = 5.4 Hz), 3.22-3.18 (m, 2H), 2.50 (t, 2H, J = 7.2 Hz), 2.06 (s, 6H), 1.87-1.82 (m, 2H), 1.74-1.68 (m, 2H), 1.55-1.48 (m, 2H), 1.47-1.41 (m, 2H), 1.38 (s, 9H), 1.31-1.24 (m, 2H); ^{13}C NMR (CD_3OD, 150 MHz): δ 20.04, 27.10, 27.76, 28.96, 29.71, 30.41, 30.98, 48.15, 48.34,

51.97, 52.94, 68.07, 69.41, 70.94, 71.39, 71.82, 81.11, 107.46, 108.8, 108.87, 115.40, 133.34, 143.46, 157.60, 161.78, 161.92, 168.50. HRMS (DART) *m/z:* calcd for $C_{33}H_{52}N_6O_8$ + H^+ 661.3920; found 661.3929 $(M + H)^+$.

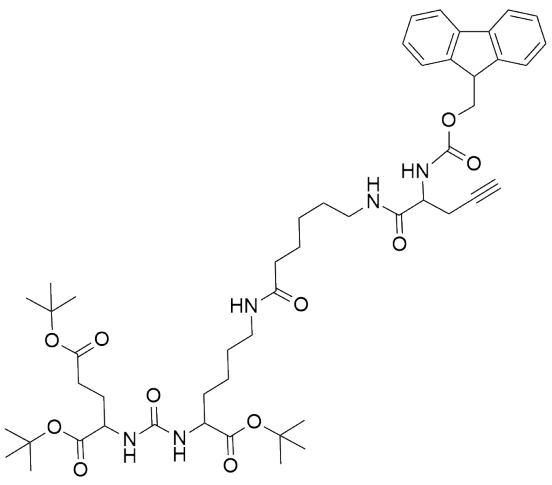


16

3-((6-((2-(2-(2-azidoethoxy)ethoxy)ethyl)(*tert*-butoxycarbonyl)amino)hexyl)oxy)-5-(3-(3,5-dimethyl-1*H*-pyrazol-4-yl)propoxy)benzoic acid (16). To a solution of **33** (400 mg, 0.606 mmol, 1 equiv) in a mixture of MeOH (4 ml) and water (1 ml) was added NaOH (48.4 mg, 1.21 mmol, 2 equiv). The reaction mixture was heated at 50°C for 14 h and then concentrated under reduced pressure. The residue was extracted with 1% acetic acid in 50% MeOH/EtOAc. The combined organic extracts were concentrated under reduced pressure and the product was subject to purification by preparative HPLC to afford compound **16** (294 mg, 75% yield); ^{1}H NMR (CD_3OD, 600 MHz) δ 7.05-7.01 (m, 2H), 6.56 (t, 1H, J = 2.4 Hz), 3.88 (t, 2H, J = 6 Hz), 3.81 (t, 2H, J = 6 Hz), 3.56-3.53 (m, 2H), 3.53-3.49 (m, 4H), 3.49-3.46 (m, 2H), 3.27 (t, 2H, J = 5.4 Hz), 3.25-3.22 (m, 2H), 3.17 (t, 2H, J = 7.2 Hz), 2.48 (t, 2H, J = 7.2 Hz), 2.04 (s, 6H), 1.85-1.79 (m, 2H), 1.72-1.65 (m, 2H), 1.52-1.44 (m, 2H), 1.44-1.38 (m, 2H), 1.34 (s, 9H), 1.28-1.22 (m, 2H); ^{13}C NMR (CD_3OD, 150 MHz): δ 10.76, 20.04, 27.11, 27.76, 28.96, 29.217, 30.42, 31.0, 48.15, 48.34, 51.97, 68.03, 69.37, 70.90, 71.39, 71.82, 81.12, 107.27, 109.01, 109.08, 115.47, 134.23, 143.48, 157.61, 161.70, 161.86, 169.94. HRMS (DART) *m/z:* calcd for $C_{32}H_{50}N_6O_8$ + H^+ 647.3763; found 647.3775 $(M + H)^+$.

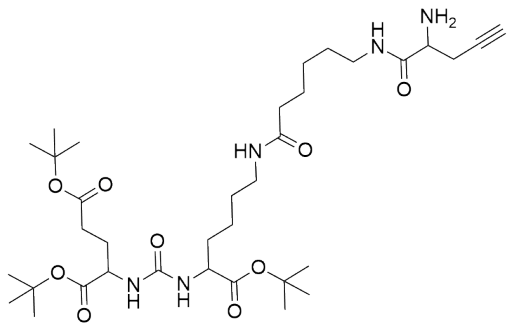


Scheme S4. Synthesis of PSMA ligand **22** and intermediates **10** and **15**. a) Fmoc-L-propargylglycine, HOBt monohydrate, EDCI, DIPEA, DCM, rt, 16 h. b) DMF, piperidine (1:1), rt, 3 h. c) 20% TFA in DCM, rt, 3 h. d) 3-Tritylthio propionic acid, HOBt monohydrate, EDCI, DIPEA, DCM, rt, 16 h.



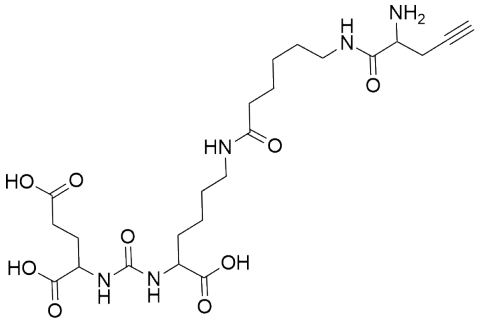
35

tri-tert-butyl 1-(9H-fluoren-9-yl)-3,6,13,21-tetraoxo-5-(prop-2-yn-1-yl)-2-oxa-4,7,14,20,22-pentaazapentacosane-19,23,25-tricarboxylate (35). To a solution of **34** (synthesized as reported earlier)[4] (9.4 g, 15.6 mmol, 1 equiv) in DCM (100 ml) was added Fmoc-L-propargylglycine (5.8 g, 17.16 mmol, 1.1 equiv), HOBt monhydrate (2.39 g, 15.6 mmol, 1 equiv), EDCI (2.99 g, 15.6 mmol, 1 equiv), and DIPEA (7.73 ml, 46.8 mmol, 3 equiv). The reaction was flushed with nitrogen and stirred overnight. The crude reaction mixture was then diluted with DCM (350 ml) and washed with water (3×100 ml). The DCM fraction was concentrated under reduced pressure and the residue was purified by flash column chromatography (silica gel, 1-10% EtOAc/hexanes) to afford compound **35** (11 g, 76% yield); ^{1}H NMR (CD_3OD, 600 MHz) δ 7.76 (d, 2H, J = 7.8 Hz), 7.64 (d, 2H, J = 7.8 Hz), 7.36 (t, 2H, J = 7.8 Hz), 7.28 (t, 2H, J = 7.8 Hz), 4.39-4.29 (m, 2H), 4.23-4.10 (m, 4H), 3.22-3.09 (m, 4H), 2.68-2.53 (m, 2H), 2.33-2.23 (m, 3H), 2.13 (t, 2H, J = 7.8 Hz), 2.05-1.98 (m, 1H), 1.80-1.70 (m, 2H), 1.62-1.26 (m, 11H), 1.45-1.41 (m, 27H); ^{13}C NMR (CD_3OD, 150 MHz): δ 21.72, 22.63, 25.33, 26.13, 26.97, 27.00, 27.04, 27.72, 28.66, 28.70, 31.90, 35.67, 38.75, 39.00, 47.04, 52.83, 53.50, 54.13, 66.80, 71.00, 79.09, 80.41, 81.25, 81.48, 119.63, 124.91, 124.96, 126.86, 127.50, 141.27, 143.86, 143.91, 156.86, 158.60, 171.31, 172.17, 172.42, 172.61, 174.67. HRMS (DART) *m/z:* calcd for $C_{50}H_{71}N_5O_{11}$ + H^+ 918.5223; found 918.5183 (M + H)$^+$.



10

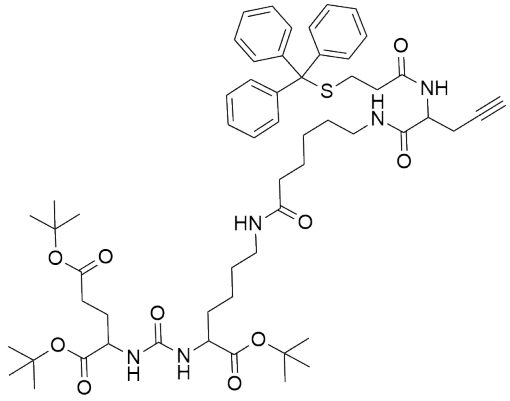
tri-tert-butyl 21-amino-5,13,20-trioxo-4,6,12,19-tetraazatetracos-23-yne-1,3,7-tricarboxylate (10). To a solution of **35** (9 g, 9.8 mmol, 1 equiv) in DMF (40 ml), was added piperidine (40 ml) and the reaction was stirred at room temperature for 3 h. The solution was concentrated under reduced pressure and the residue was purified by flash column chromatography (silica gel, 1-100% EtOAc/hexanes and then 5% MeOH/EtOAc) to afford compound **10** (5.93 g, 87% yield); ^{1}H NMR (CD_3OD, 600 MHz) δ 4.18-4.16 (m, 1H), 4.12-4.09 (m, 1H), 3.84 (t, 1H, J = 6 Hz), 3.27-3.16 (m, 2H), 3.14 (t, 2H, J = 6 Hz), 2.78-2.69 (m, 2H), 2.60 (t, 1H, J = 2.4 Hz), 2.34-2.25 (m, 2H), 2.16 (t, 2H, J = 7.2 Hz), 2.05-1.99 (m, 1H), 1.81-1.71 (m, 2H), 1.63-1.30 (m, 11H), 1.46-1.42 (m, 27H). ^{13}C NMR (CD_3OD, 150 MHz): δ 21.77, 22.66, 26.13, 26.95, 26.98, 27.01, 27.70, 28.60, 28.64, 31.15, 31.88, 35.58, 38.75, 39.11, 52.01, 52.84, 53.52, 73.16, 76.66, 80.43, 81.26, 81.48, 158.62, 166.36, 168.36, 172.15, 172.43, 172.61. HRMS (DART) *m/z:* calcd for $C_{35}H_{61}N_5O_9$ + H^+ 696.4542; found 696.4552 $(M + H)^+$.



22

21-amino-5,13,20-trioxo-4,6,12,19-tetraazatetracos-23-yne-1,3,7-tricarboxylic acid (22). To a solution of **10** (50 mg, 0.072 mmol, 1 equiv) in DCM (3.2 ml) was added TFA (0.8 ml). The reaction was stirred at room temperature for 3 h and then dried under vacuum. The crude reaction mixture was then purified using preparative HPLC to afford **22** (22.7 mg, 60% yield); (97% purity

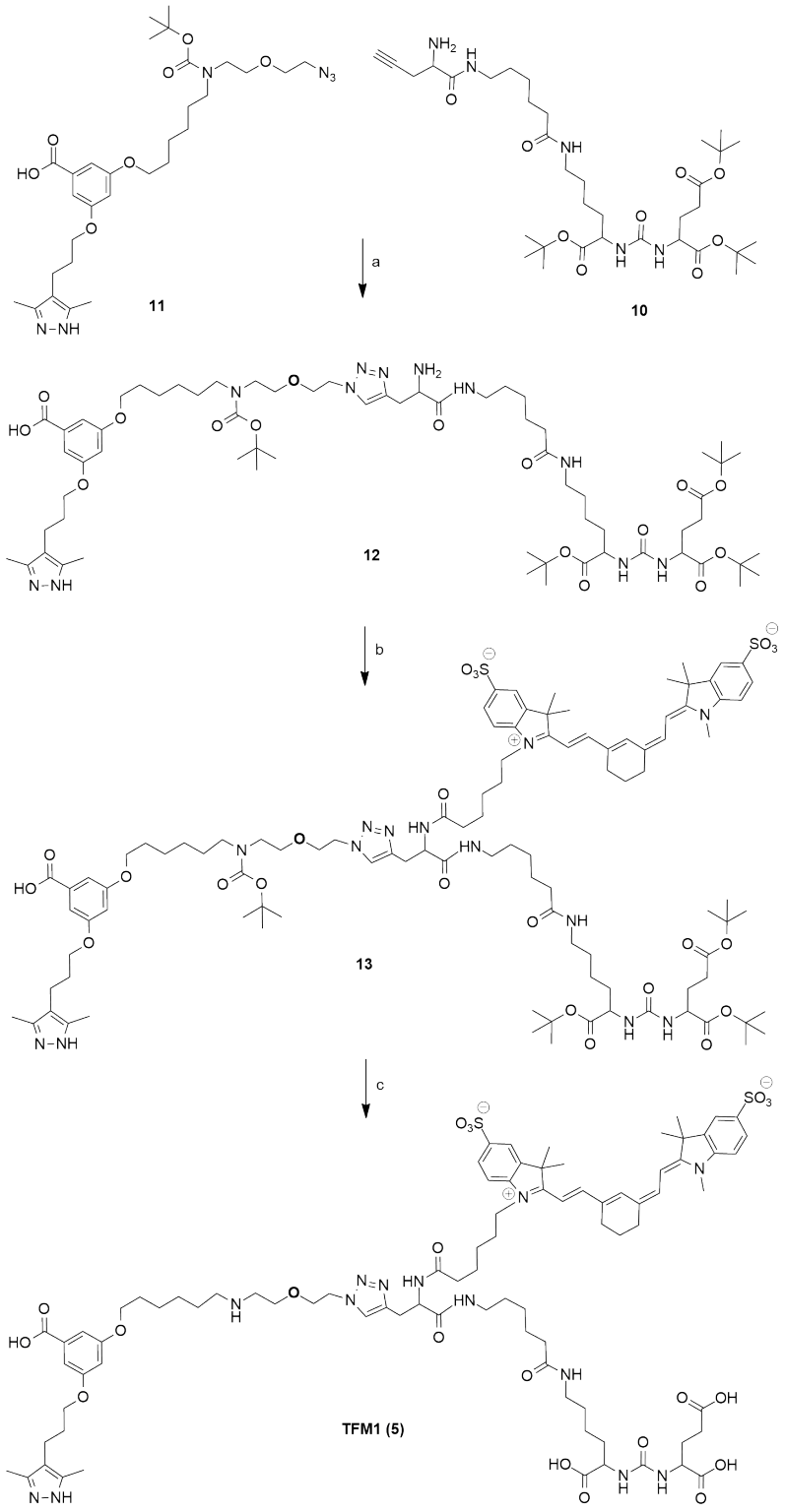
by HPLC): t_R (column) (C18) = 10.1 min; t_R (C4) = 5.4 min.^{1}H NMR (CD_3OD, 600 MHz) δ 4.23-4.19 (m, 2H), 3.94 (t, 1H, *J* = 6 Hz), 3.27-3.21 (m, 2H), 3.15 (t, 2H, *J* = 6 Hz), 2.81-2.73 (m, 2H), 2.60 (t, 1H, *J* = 2.4 Hz), 2.42-2.32 (m, 2H), 2.16 (t, 2H, *J* = 7.2 Hz), 2.13-2.07 (m, 1H), 1.92-1.78 (m, 2H), 1.66-1.47 (m, 7H), 1.42-1.31 (m, 4H). ^{13}C NMR (CD_3OD, 150 MHz): δ 21.36, 22.60, 25.16, 25.91, 28.28, 28.46, 28.49, 30.26, 32.25, 35.49, 38.75, 39.13, 51.81, 53.27, 53.37, 73.33, 76.43, 158.75, 165.75, 167.76, 175.88, 175.98, 176.24. HRMS (DART) *m/z:* calcd for $C_{23}H_{37}N_5O_9$ + H^+ 528.2664; found 528.2627 $(M + H)^+$.



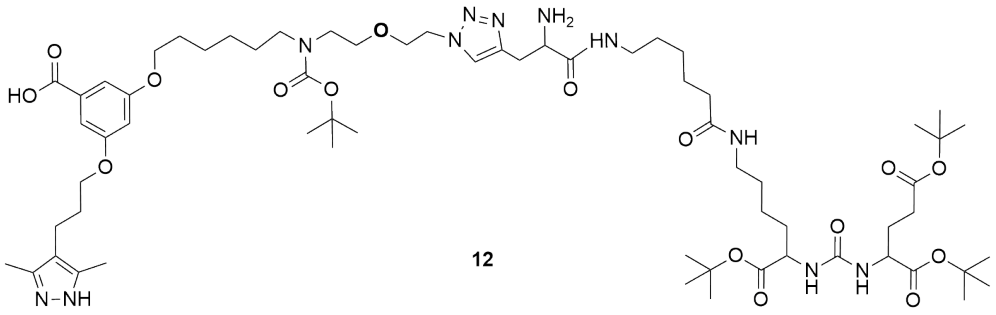
15

tri-tert-butyl 5,8,15,23-tetraoxo-1,1,1-triphenyl-7-(prop-2-yn-1-yl)-2-thia-6,9,16,22,24-pentaazaheptacosane-21,25,27-tricarboxylate (15). To a solution of **10** (1 g, 1.44 mmol, 1 equiv) in DCM (20 ml) was added 3-Tritylthio propionic acid (502 mg, 1.44 mmol, 1 equiv), HOBt monhydrate (220 mg, 1.44 mmol, 1 equiv), EDCI (276 mg, 1.44 mmol, 1 equiv), and DIPEA (0.71 ml, 4.32 mmol, 3 equiv). The reaction was flushed with nitrogen and stirred for 16 h. The crude reaction mixture was then diluted with DCM (150 ml) and washed with water (3×100 ml). The DCM fraction was dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The residue was purified by flash column chromatography (silica gel, 1-10% EtOAc/hexanes) to afford compound **15** (1.02 g, 69% yield). ^{1}H NMR (CD_3OD, 600 MHz) δ 7.37-7.35 (m, 6H), 7.27 (t, 6H, *J* = 7.8 Hz), 7.19 (t, 3H, *J* = 7.8 Hz), 4.39 (t, 1H, *J* = 7.2 Hz), 4.19-4.16 (m, 1H), 4.12-4.10 (m, 1H), 3.15-3.07 (m, 4H), 2.64-2.51 (m, 2H), 2.46-2.39 (m, 2H), 2.32-2.27 (m, 3H), 2.23 (t, 2H, *J* = 7.2 Hz), 2.12 (t, 2H, *J* = 7.2 Hz), 2.04-1.99 (m, 1H), 1.81-1.71 (m, 2H), 1.63-1.30 (m, 11H), 1.46-1.42 (m, 27H); ^{13}C NMR (CD_3OD, 150 MHz): δ 21.30, 22.64, 25.32, 26.11, 26.97, 27.00, 27.03, 27.65, 27.71, 28.70, 31.16, 31.89, 34.28, 35.69, 37.38, 38.76,

38.96, 52.42, 52.83, 53.50, 66.51, 71.01, 78.96, 80.42, 81.25, 81.47, 126.53, 127.64, 129.40, 144.80, 158.61, 162.42, 170.77, 172.16, 172.42, 172.55, 174.67. HRMS (DART) *m/z:* calcd for $C_{57}H_{79}N_5O_{10}S + H^+$ 1026.5621; found 1026.5503 $(M + H)^+$.

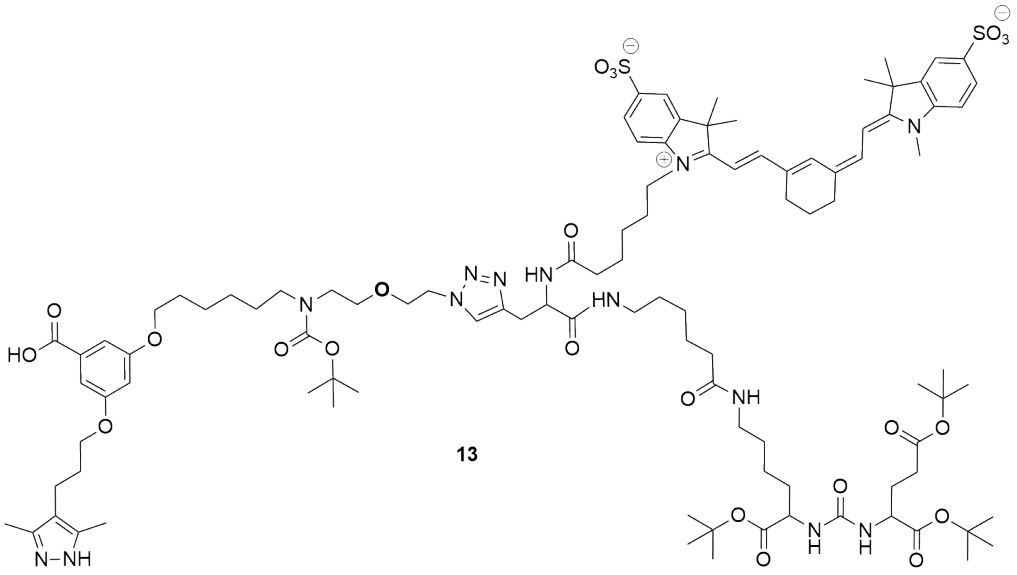


Scheme S5. Synthesis of TFM1 (**5**). a) $CuSO_4$, sodium ascorbate, of H_2O/THF (2:1), rt, 3 h; b) Sulfo-Cyanine7 NHS ester, DIPEA, DMF, rt, 16 h; c) TFA, TIS, and CH_2Cl_2 (50:3:100 ratio), rt, 2 h.



12

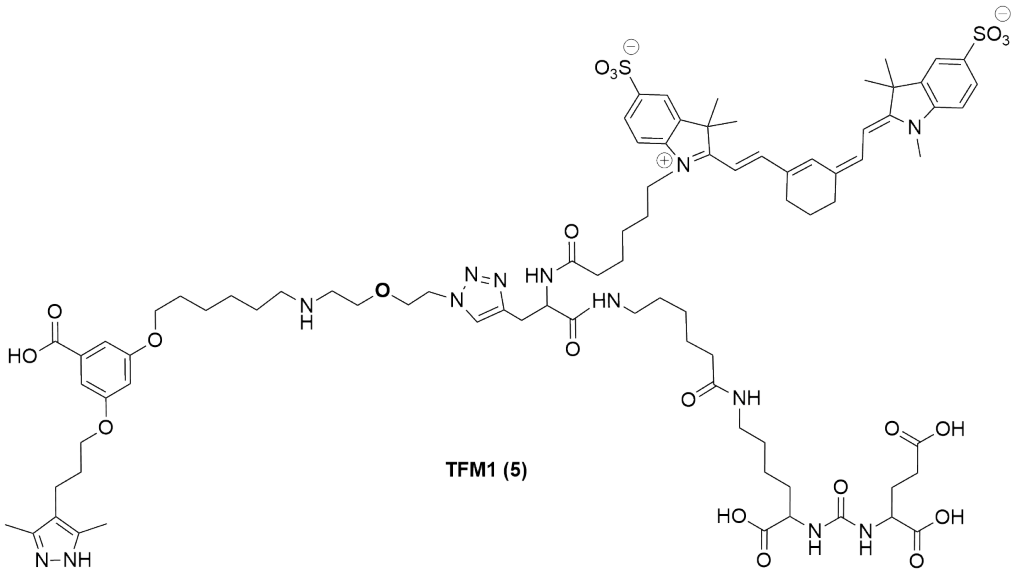
Synthesis of 25. The click (CuAAC) was carried out by reacting **10** (150 mg, 0.216 mmol, 1 equiv) with **11** (130 mg, 0.216 mmol, 1 equiv), and $CuSO_4$ pentahydrate (14 mg, 0.054 mmol, 0.25 equiv), and sodium ascorbate (21 mg, 0.108 mmol, 0.5 equiv) in a mixture of H_2O/THF (2:1) (3 ml). The reaction mixture was stirred at room temperature overnight. The solution was concentrated under reduced pressure. The residue was extracted with 10% MeOH/EtOAc. The combined organic extracts were concentrated under reduced pressure and purified by preparative HPLC to afford compound **12** (129 mg, 46% yield). ESI-MS: Exact mas calcd for $C_{65}H_{107}N_{11}O_{16}$ $[M+H]^+$ 1298.8; $[M+Na]^+$ 1320.8; $[M+2H]^{2+}$ 649.9; $[M+H+Na]^{2+}$ 660.9. Found: 1299.0, 1321.1, 650.2, 661.3]



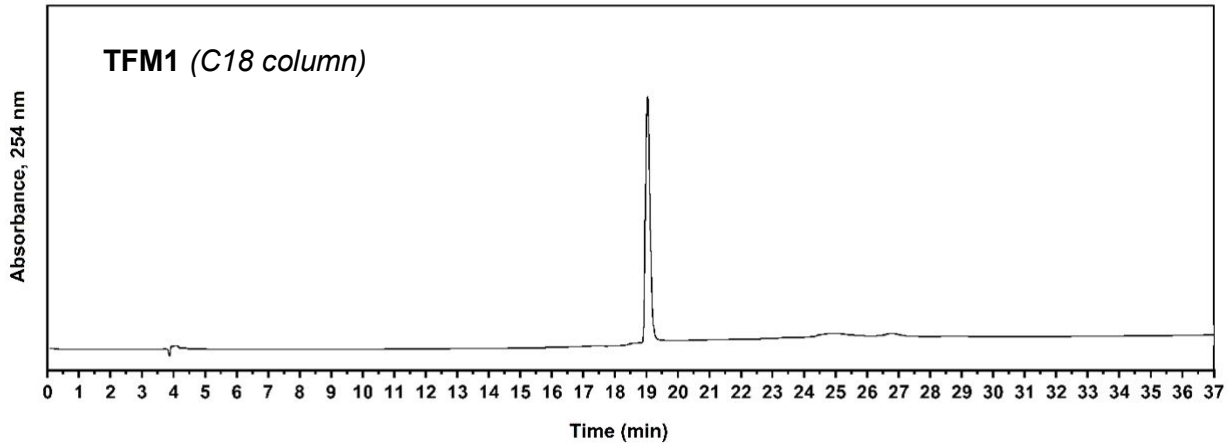
13

Synthesis of 13. To a solution of **12** (50 mg, 0.039 mmol, 1 equiv) and the amine reactive succinimide ester (sulfo-Cyanine7) [sodium 1-(6-((2,5-dioxopyrrolidin-1-yl)oxy)-6-oxohexyl)-3,3-dimethyl-2-((E)-2-((Z)-3-((Z)-2-(1,3,3-trimethyl-5-sulfonatoindolin-2-

ylidene)ethylidene)cyclohex-1-en-1-yl)vinyl)-3H-indol-1-ium-5-sulfonate] (32 mg, 0.039 mmol, 1 equiv) in anhydrous DMF (3 ml), was added DIPEA (0.064 ml, 0.39 mmol, 10 equiv). The reaction mixture was stirred at room temperature for 16 h. The solution was concentrated under reduced pressure and purified by preparative HPLC to afford compound **13** (48 mg, 62% yield); Exact mas calcd for $C_{102}H_{149}N_{13}O_{23}S_2$ $[M+H]^+$ 1989.0; $[M+Na]^+$ 2011.0 $[M+2H]^{2+}$ 995.0. Found: 1989.3, 2011.4, 996.0.



Synthesis of TFM1 (5). To a solution of **13** (20 mg, 0.010 mmol, 1 equiv) was added a mixture containing TFA and CH_2Cl_2, (1:9 ratio) (2 ml) and the reaction was stirred at room temperature for 3 h. The solution was concentrated under reduced pressure and purified by preparative HPLC to afford compound TFM1 (**5**) (15 mg, 87% yield); (97% purity by HPLC): t_R (column) (C18) = 19 min; t_R (C4) = 14.5 min; ESI-MS: Exact mas calcd for $C_{85}H_{117}N_{13}O_{21}S_2$ $[M+H]^+$ 1720.8; $[M+Na]^+$ 1742.8; $[M+H]^{2+}$ 860.9; $[M+H+Na]^{2+}$ 871.9; $[M+2Na-H]^+$ 1764.8. Found: 1721.3, 1743.3, 861.5, 872.6, 1765.4.


TFM1 (C18 column)
Absorbance, 254 nm
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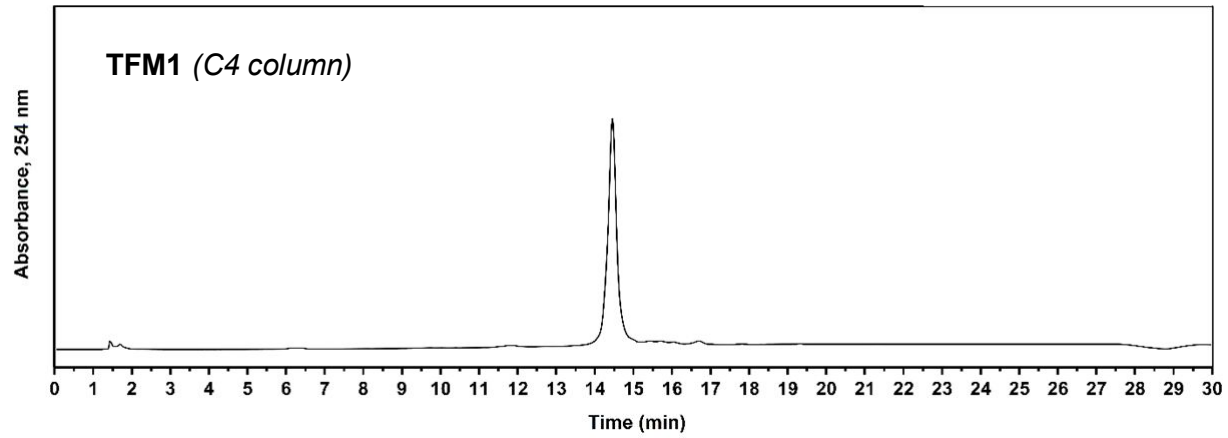
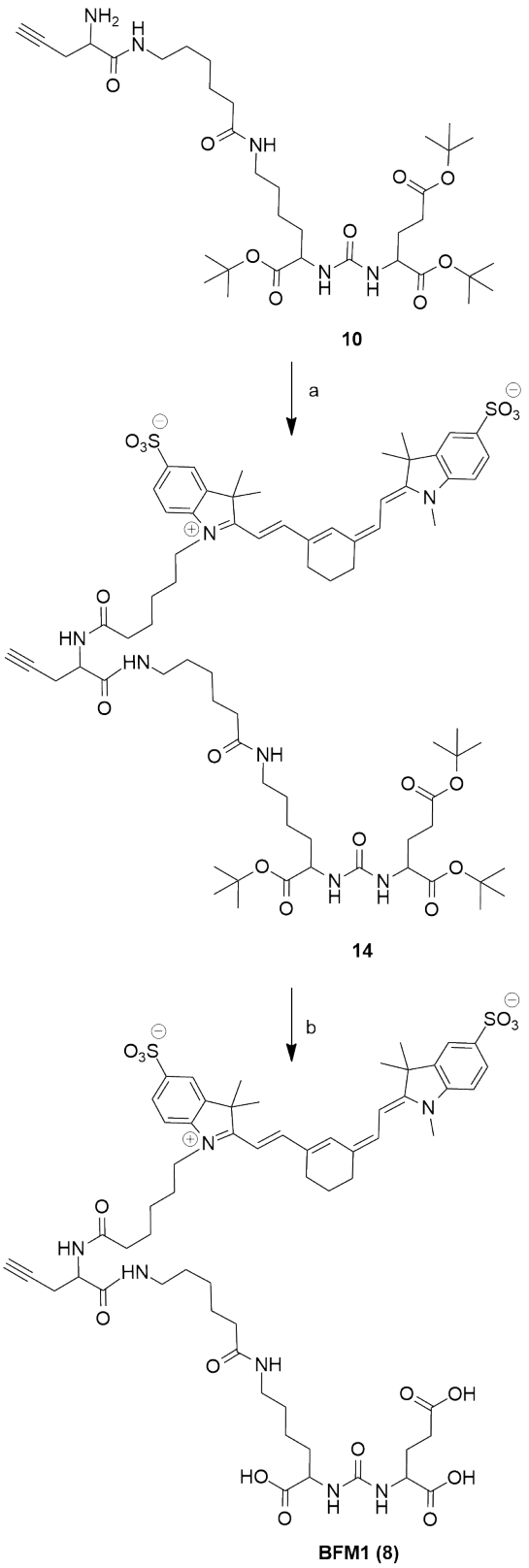
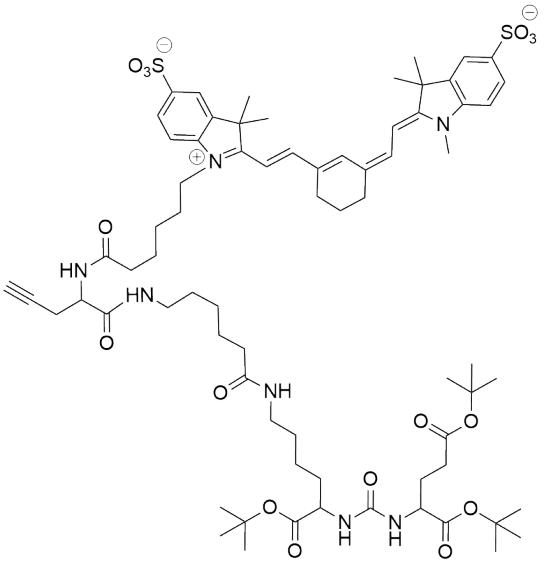
Time (min)


TFM1 (C4 column)
Absorbance, 254 nm
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Time (min)

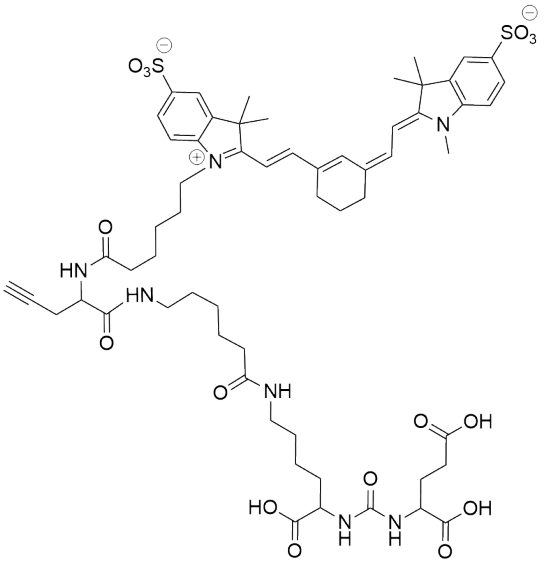


Scheme S6. Synthesis of BFM1 (**8**). a) Sulfo-Cyanine7 NHS ester, DIPEA, DMF, rt, 16 h; b) TFA, TIS, and CH_2Cl_2 (50:3:100 ratio), rt, 2 h.



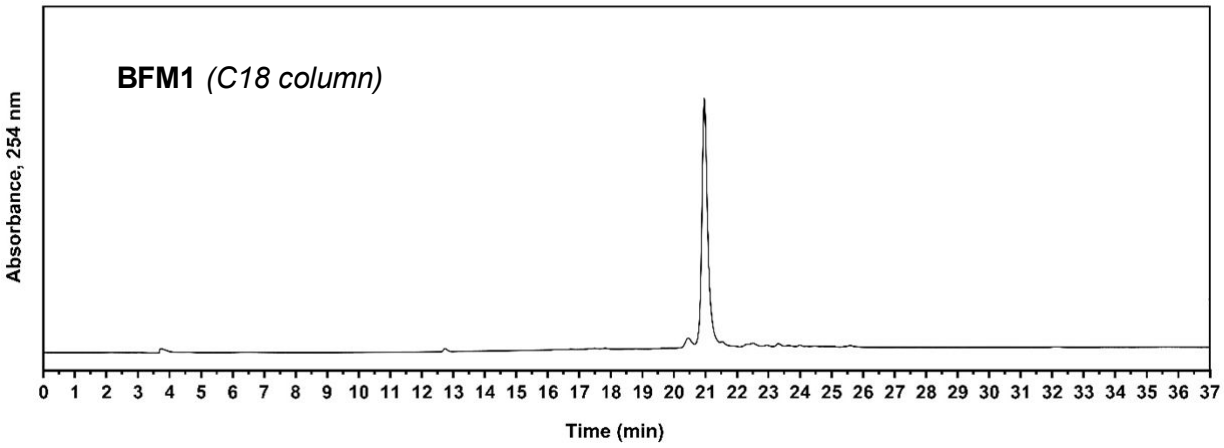
14

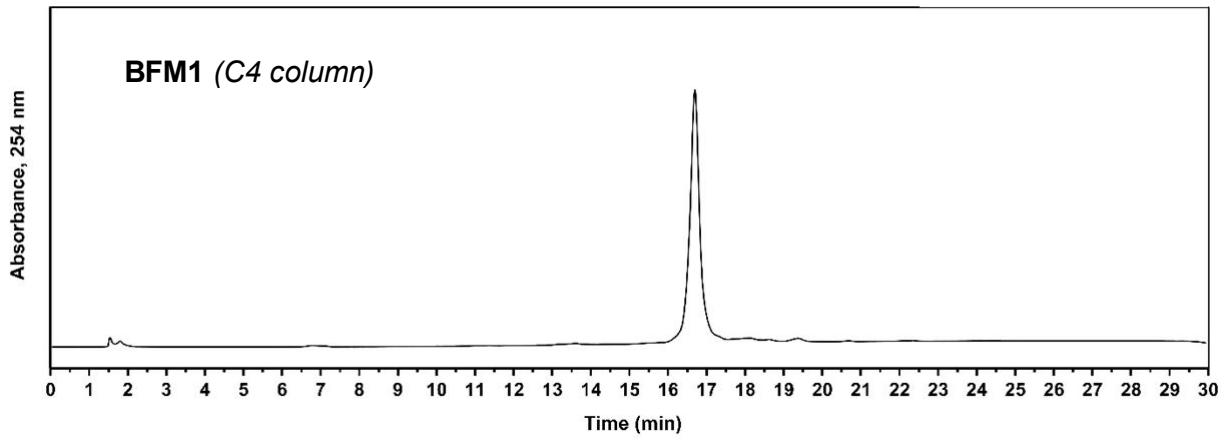
Synthesis of 14. To a solution of **10** (30 mg, 0.043 mmol, 1 equiv) and the amine reactive succinimide ester (sulfo-Cyanine7) (36 mg, 0.0043 mmol, 1 equiv) in anhydrous DMF (3 ml), was added DIPEA (0.075 ml, 0.43 mmol, 10 equiv). The reaction mixture was stirred at room temperature for 16 h. The solution was concentrated under reduced pressure and purified by preparative HPLC to afford compound **14** (33 mg, 55% yield); Exact mas calcd for $C_{72}H_{103}N_7O_{16}S_2$ $[M+H]^+$ 1386.7; $[M+Na]^+$ 1408.7; $[M+2H]^{2+}$ 693.9; $[M+H+Na]^{2+}$; 704.3. Found: 1387.0, 1409.0, 694.1, 705.2.

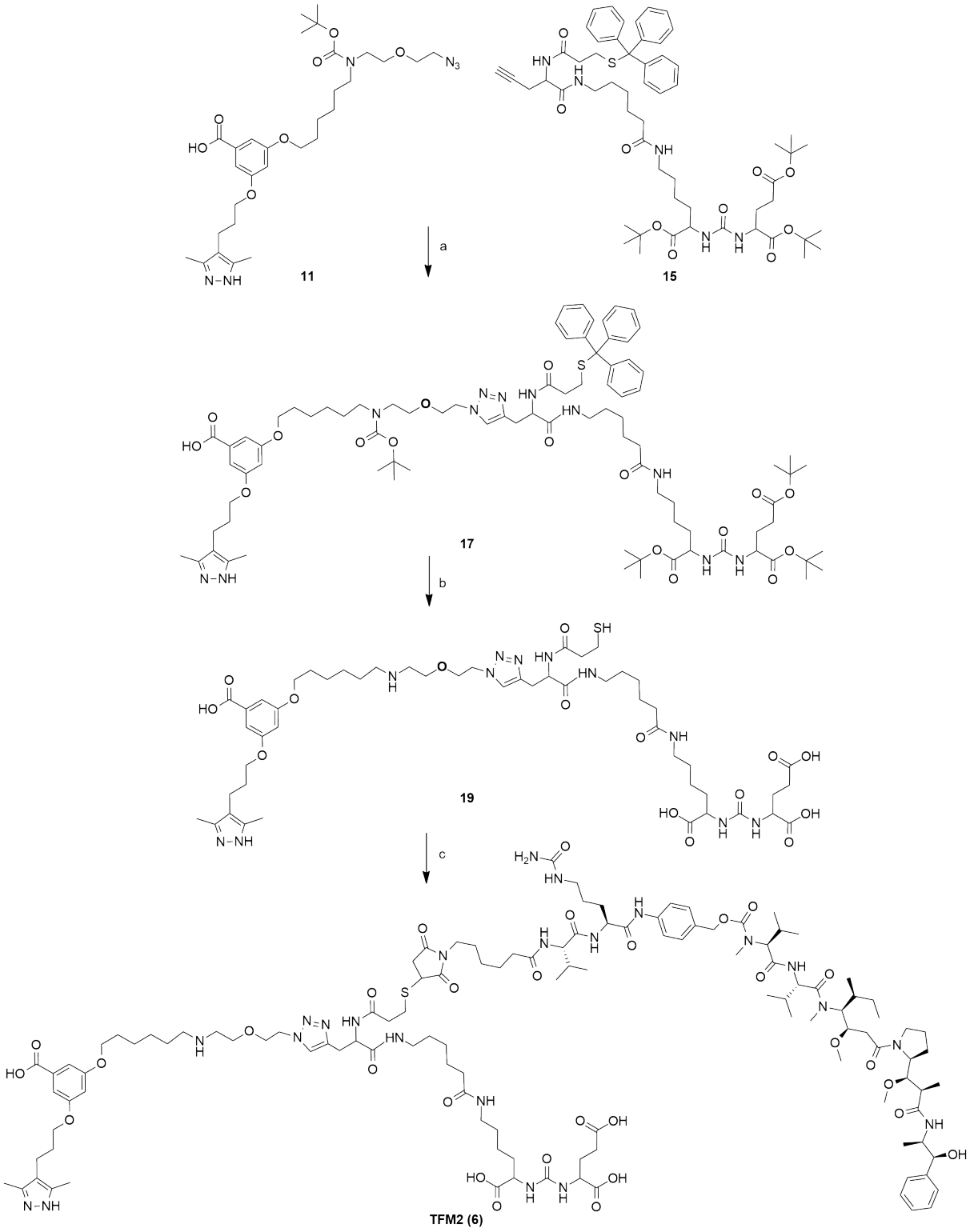


BFM1 (8)

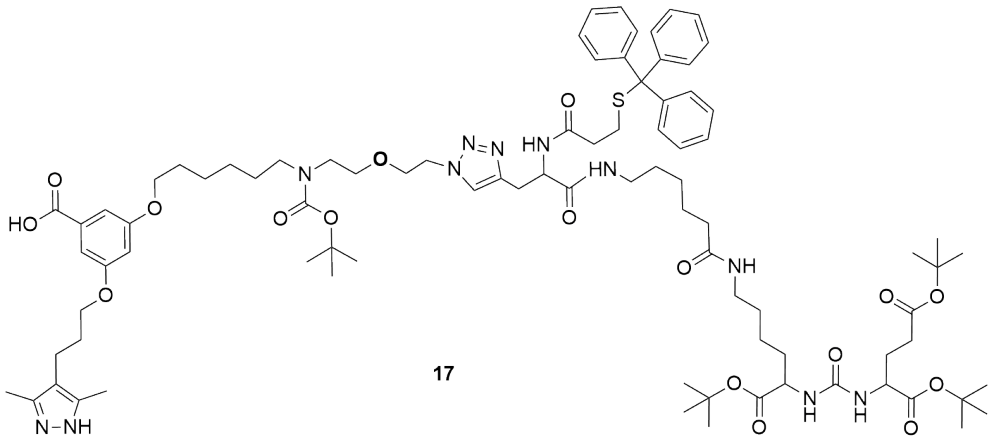
Synthesis of BFM1 (8). To a solution of **14** (20 mg, 0.014 mmol, 1 equiv) was added a mixture of TFA and CH_2Cl_2, (1:9 ratio) (2 ml) and the reaction was stirred at room temperature for 3 h. The solution was concentrated under reduced pressure and purified by preparative HPLC to afford compound BFM1 (**8**) (14 mg, 81% yield); (95% purity by HPLC): t_R (column) (C18) = 21 min; t_R (C4) = 16.7 min; ESI-MS: Exact mas calcd for $C_{60}H_{79}N_7O_{16}S_2$ $[M+H]^+$ 1218.5. Found: 1218.7.





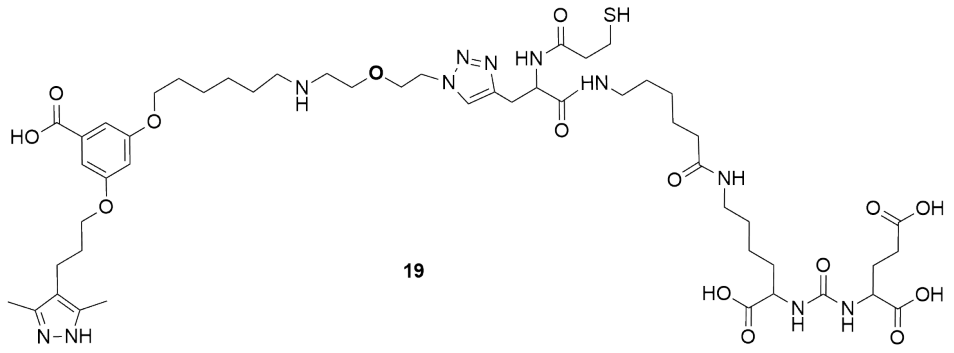


Scheme S7. Synthesis of TFM2 (**6**). a) $CuSO_4$, sodium ascorbate, of H_2O/THF (2:1), rt, 3 h; b) TFA, TIS and CH_2Cl_2 (10: 10: 1 ratio); c) VcMMAE, TEA, DMF, rt, 5 h.



17

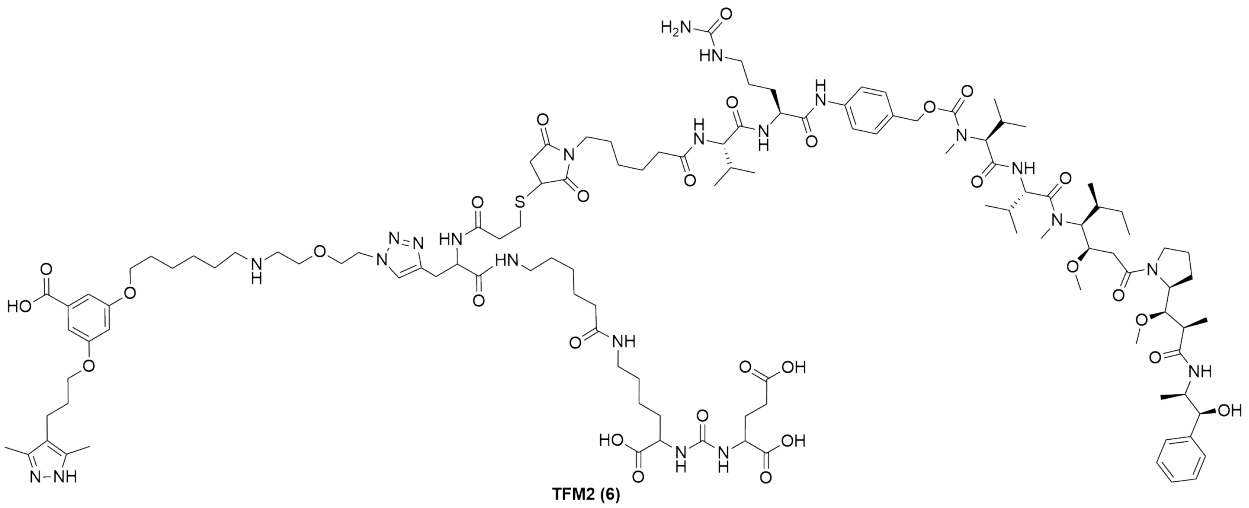
Synthesis of 17: The click (CuAAC) coupling was carried out by reacting **15** (103 mg, 0.1 mmol, 1 equiv), **11** (60 mg, 0.1 mmol, 1 equiv), $CuSO_4$ (6.1 mg, 0.025 mmol, 0.25 equiv), and sodium ascorbate (16 mg, 0.05 mmol, 0.5 equiv) in a mixture of H_2O/THF (2:1) (3 ml). The reaction mixture was stirred at room temperature for 3 h. The solution was concentrated under reduced pressure. The residue was extracted with 20% MeOH/EtOAc. The combined organic extracts were concentrated under reduced pressure and purified by flash column chromatography (silica gel, 1-40% MeOH/EtOAc) to afford compound **17** (75 mg, 46% yield). ESI-MS: Exact mas calcd for $C_{87}H_{125}N_{11}O_{17}S$ $[M+H]^+$ 1628.9 $[M+Na]^+$ 1650.9; $[M+2H]^{2+}$ 815.0. Found: 1628.0, 1651.8, 815.2.



19

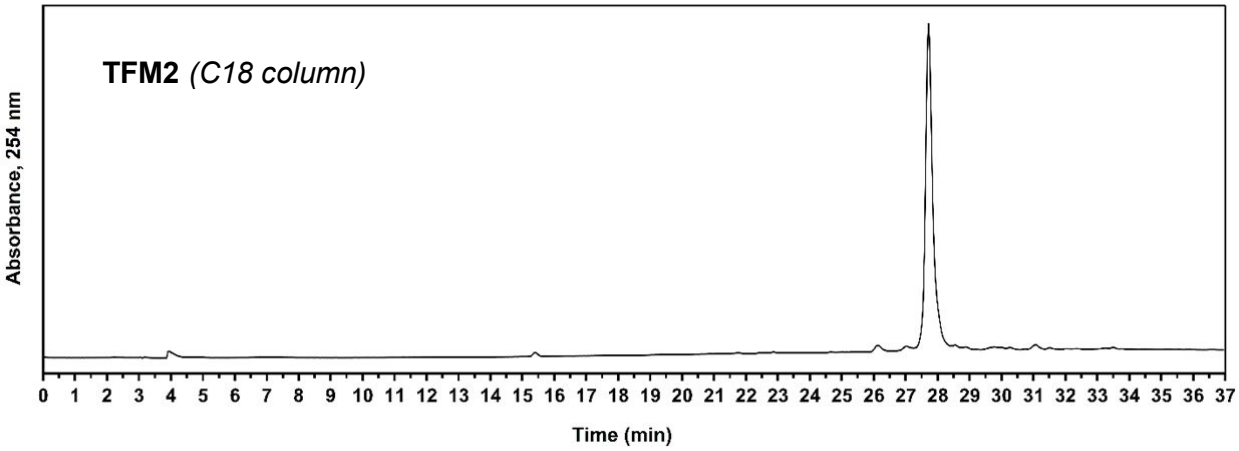
Synthesis of 19. To a solution of **17** (33 mg, 0.02 mmol, 1 equiv), a mixture of TFA, TIS, and CH_2Cl_2 (10: 10: 1 ratio) (2 ml) was added and the reaction was stirred at room temperature for 2 h. The solution was concentrated under reduced pressure. The residue was washed with CH_2Cl_2 (3x10 ml), and extracted with 20% MeOH/EtOAc. The combined organic extracts were concentrated under reduced pressure and purified by preparative HPLC to afford compound **19**;

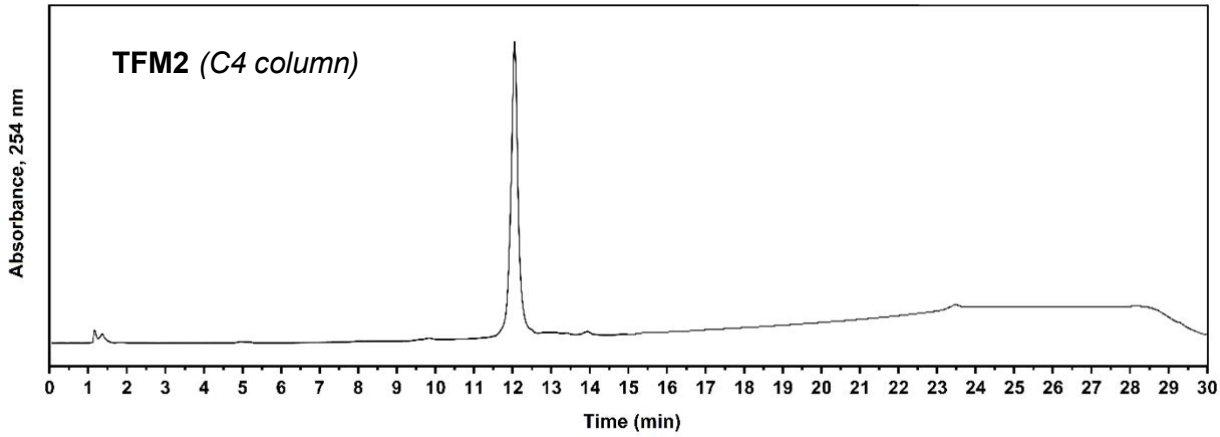
(20 mg, 88% yield). ESI-MS: Exact mas calcd for $C_{51}H_{79}N_{11}O_{15}S$ $[M+H]^+$ 1118.6; $[M+2H]^{2+}$ 559.8. Found: 1117.8, 559.5.

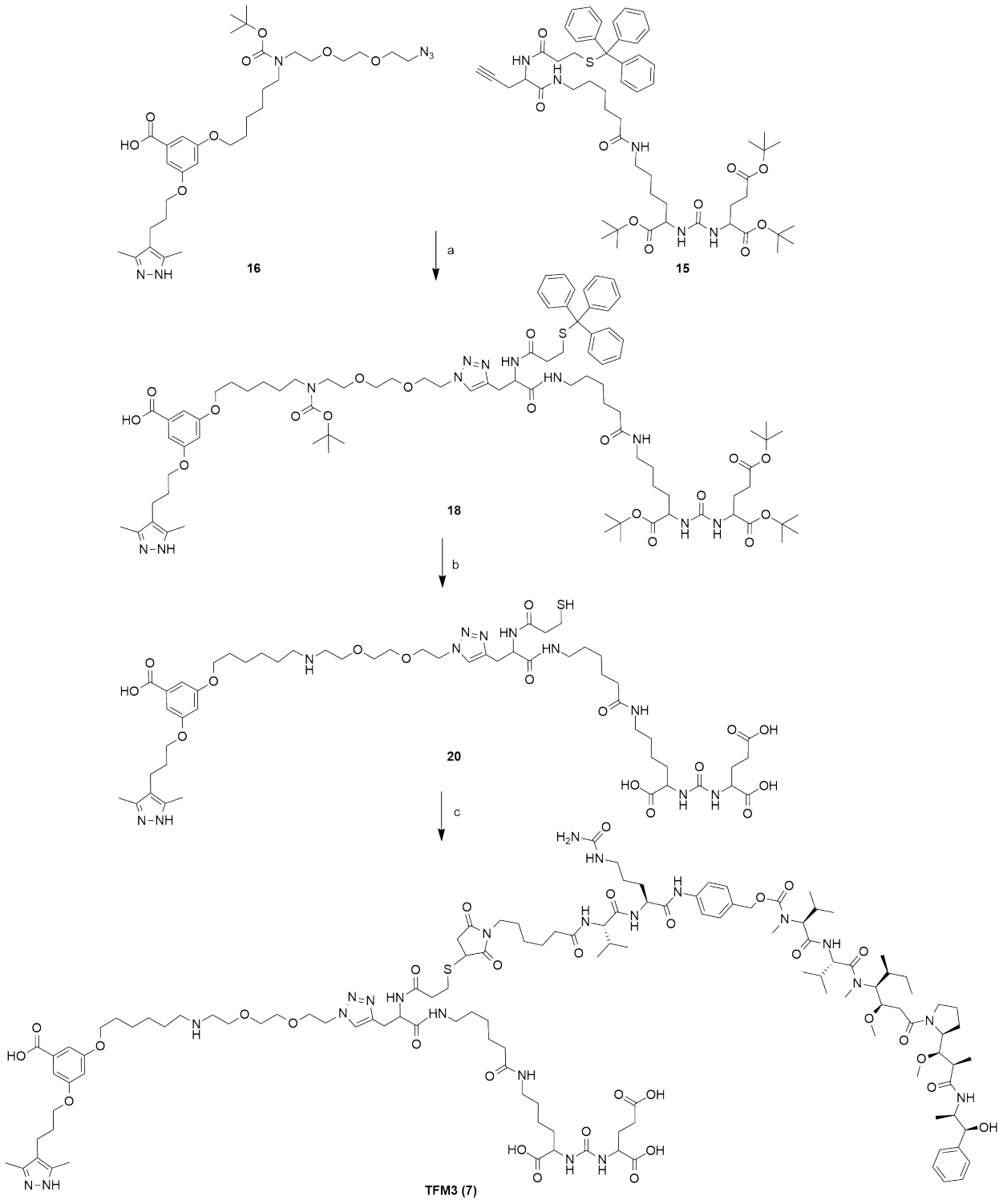


TFM2 (6)

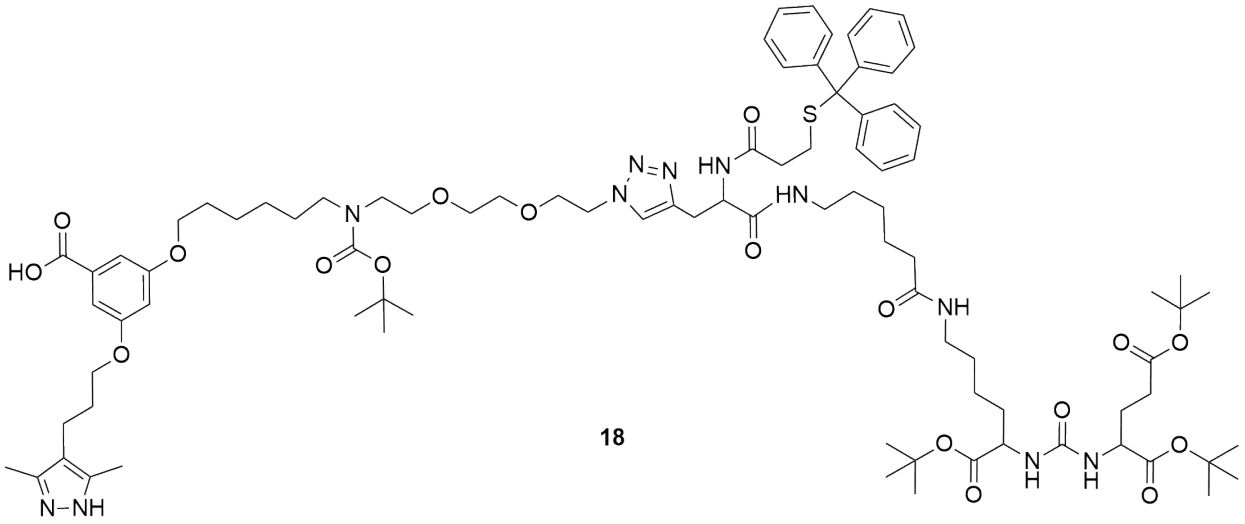
Synthesis of TFM2 (6). The maleimide-thiol conjugation reaction was carried out by reacting **19** (17 mg, 0.015 mmol, 1 equiv) with VcMMAE (14 mg, 0.01 mmol, 0.7 equiv) and TEA (50 µl) in anhydrous DMF (1.5 ml). The reaction mixture was stirred at room temperature for 5 h. The solution was concentrated under reduced pressure. The residue was washed with CH_2Cl_2 (3x10 ml) to remove any unreacted VcMMAE. The residue was extracted with 20% MeOH/CH_2Cl_2. The combined organic extracts were concentrated under reduced pressure and purified by preparative HPLC to afford TFM2 (**6**) (11 mg, 31% yield); (95% purity by HPLC): t_R (column) (C18) = 27.7 min; t_R (C4) = 12.0 min; ESI-MS: Exact mas calcd for $C_{119}H_{185}N_{22}O_{30}S$ $[M+2H]^{2+}$ 1218.2; $[M+3H]^{3+}$ 812.5; $[M+2H+Na]^{3+}$ 819.8. Found: 1218.0, 812.5, 819.9.


TFM2 (C18 column)
Absorbance, 254 nm
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37
Time (min)


TFM2 (C4 column)
Absorbance, 254 nm
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Time (min)

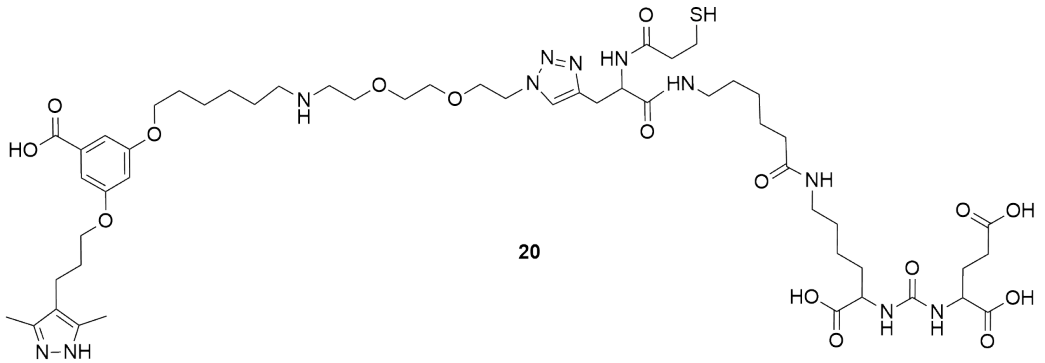


Scheme S8. Synthesis of TFM3 (**7**). a) $CuSO_4$, sodium ascorbate, of H_2O/THF (2:1), rt, 3 h; b) TFA, TIS and CH_2Cl_2 (10: 10: 1 ratio); c) VcMMAE, TEA, DMF, rt, 5 h.



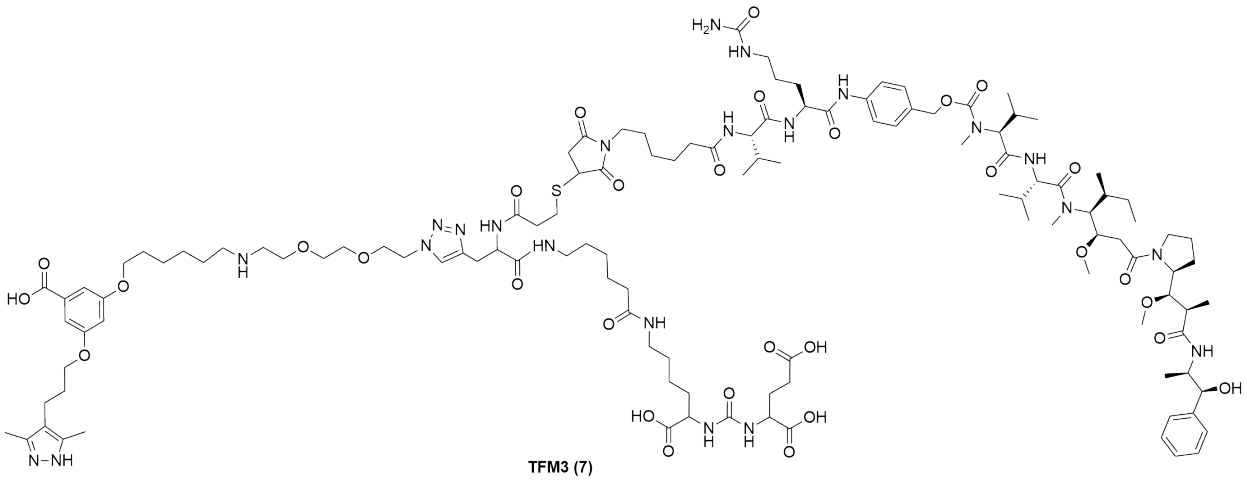
18

Synthesis of 18. The click (CuAAC) coupling was carried out by reacting **15** (167 mg, 0.163 mmol, 1 equiv), **16** (105 mg, 0.163 mmol, 1 equiv), $CuSO_4$ (10 mg, 0.041 mmol, 0.25 equiv), and sodium ascorbate (16 mg, 0.082 mmol, 0.5 equiv) in a mixture of H_2O/THF (2:1) (3 ml). The reaction mixture was stirred at room temperature for 3 h. The solution was concentrated under reduced pressure. The residue was extracted with 20% MeOH/EtOAc. The combined organic extracts were concentrated under reduced pressure and purified by flash column chromatography (silica gel, 1-40% MeOH/EtOAc) to afford compound **18** (109 mg, 40% yield). ESI-MS: Exact mas calcd for $C_{89}H_{129}N_{11}O_{18}S$ $[M+H]^+$ 1672.9; $[M+Na]^+$ 1694.9; $[M+H+Na]^{2+}$ 848.0. Found: 1673.3, 1695.4, 848.4].

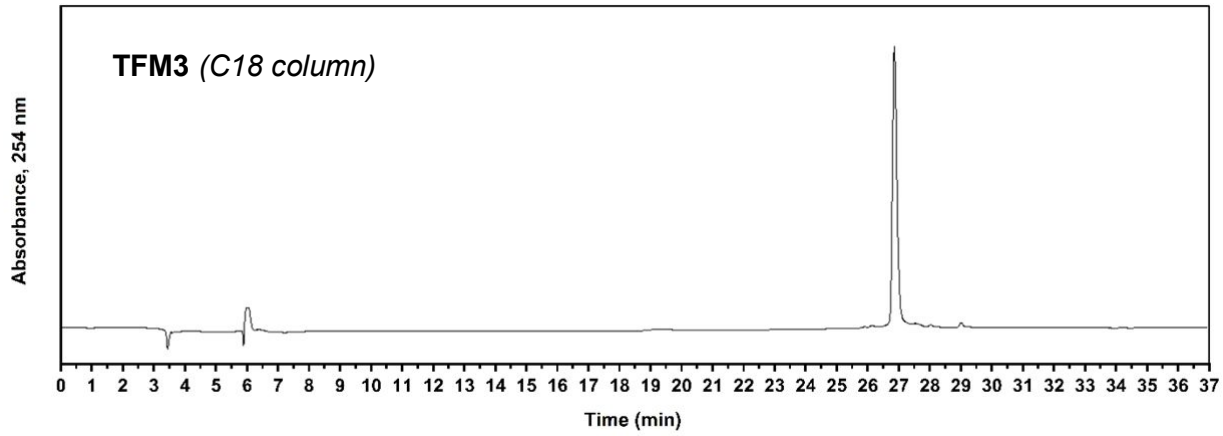


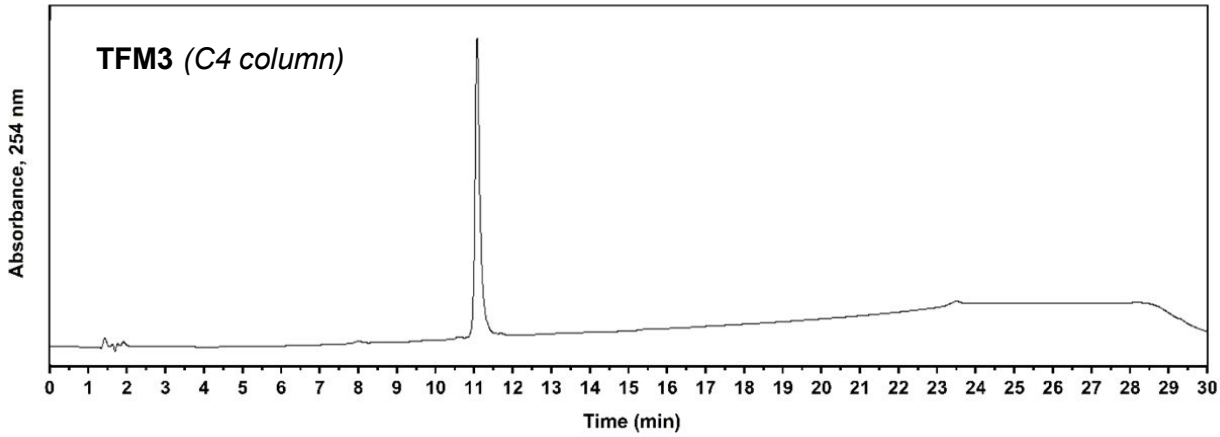
20

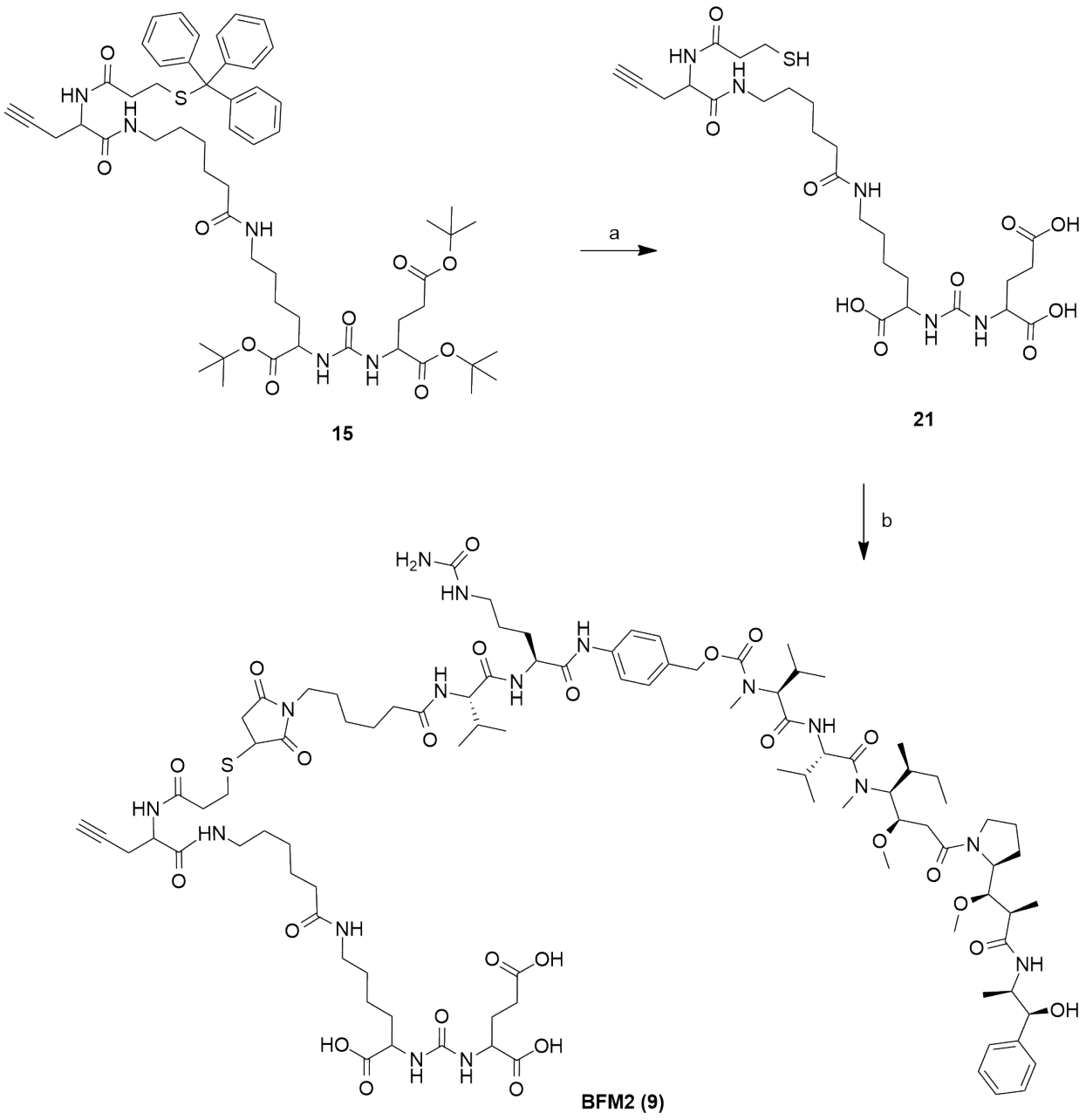
Synthesis of 20. To a solution of **18** (50 mg, 0.030 mmol, 1 equiv) a mixture of TFA, TIS, and CH_2Cl_2 (10: 10: 1 ratio) (2 ml) was added and the reaction was stirred at room temperature for 2 h. The solution was concentrated under reduced pressure. The residue was washed with CH_2Cl_2 (3x10 ml), and extracted with 20% MeOH/EtOAc. The combined organic extracts were concentrated under reduced pressure and purified by preparative HPLC to afford compound **20**; (32 mg, 92% yield). ESI-MS: Exact mas calcd for $C_{53}H_{83}N_{11}O_{16}S$ $[M+H]^+$ 1162.6; $[M+Na]^+$ 1184.6; $[M+2H]^{2+}$ 581.8; $[M+H+Na]^{2+}$ 592.8. Found: 1162.4, 1183.9, 581.6, 592.3].



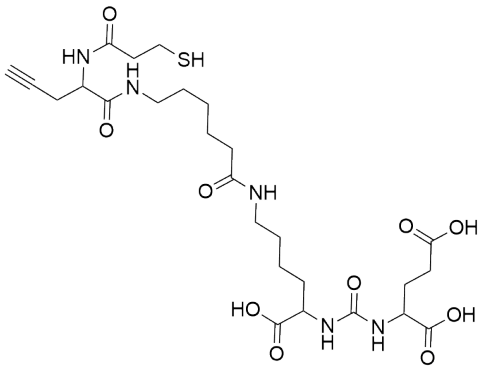
Synthesis of TFM3 (7). The maleimide-thiol conjugation reaction was carried out by reacting **20** (31.3 mg, 0.027 mmol, 1 equiv) with VcMMAE (25 mg, 0.019 mmol, 0.7 equiv) and TEA (100 µl) in anhydrous DMF (3 ml). The reaction mixture was stirred at room temperature for 5 h. The solution was concentrated under reduced pressure. The residue was washed with CH_2Cl_2 (3x10 ml) to remove any unreacted VcMMAE. The residue was extracted with 20% MeOH/CH_2Cl_2. The combined organic extracts were concentrated under reduced pressure and purified by preparative HPLC to afford TFM3 (**7**) (17 mg, 36% yield); (97% purity by HPLC): t_R (column) (C18) = 26.9 min; t_R (C4) = 11.3 min; ESI-MS: Exact mas calcd for $C_{121}H_{188}N_{22}O_{31}S$ $[M+H]^+$ 2478.4; $[M+H]^{2+}$ 1239.7; $[M+H]^{3+}$ 826.8. Found: 2478.7, 1240.8, 827.9].


TFM3 (C18 column)
Absorbance, 254 nm
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37
Time (min)


TFM3 (C4 column)
Absorbance, 254 nm
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Time (min)

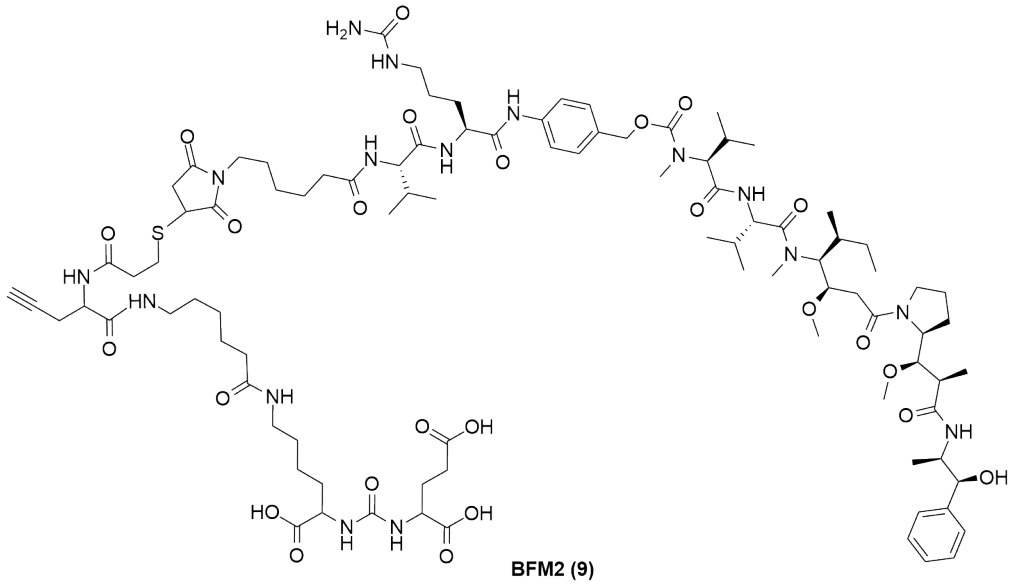


Scheme S9. Synthesis of BFM2 (**9**). a) TFA, TIS and CH_2Cl_2 (50:3:100 ratio); b) VcMMAE, TEA, DMF, rt, 5 h.



21

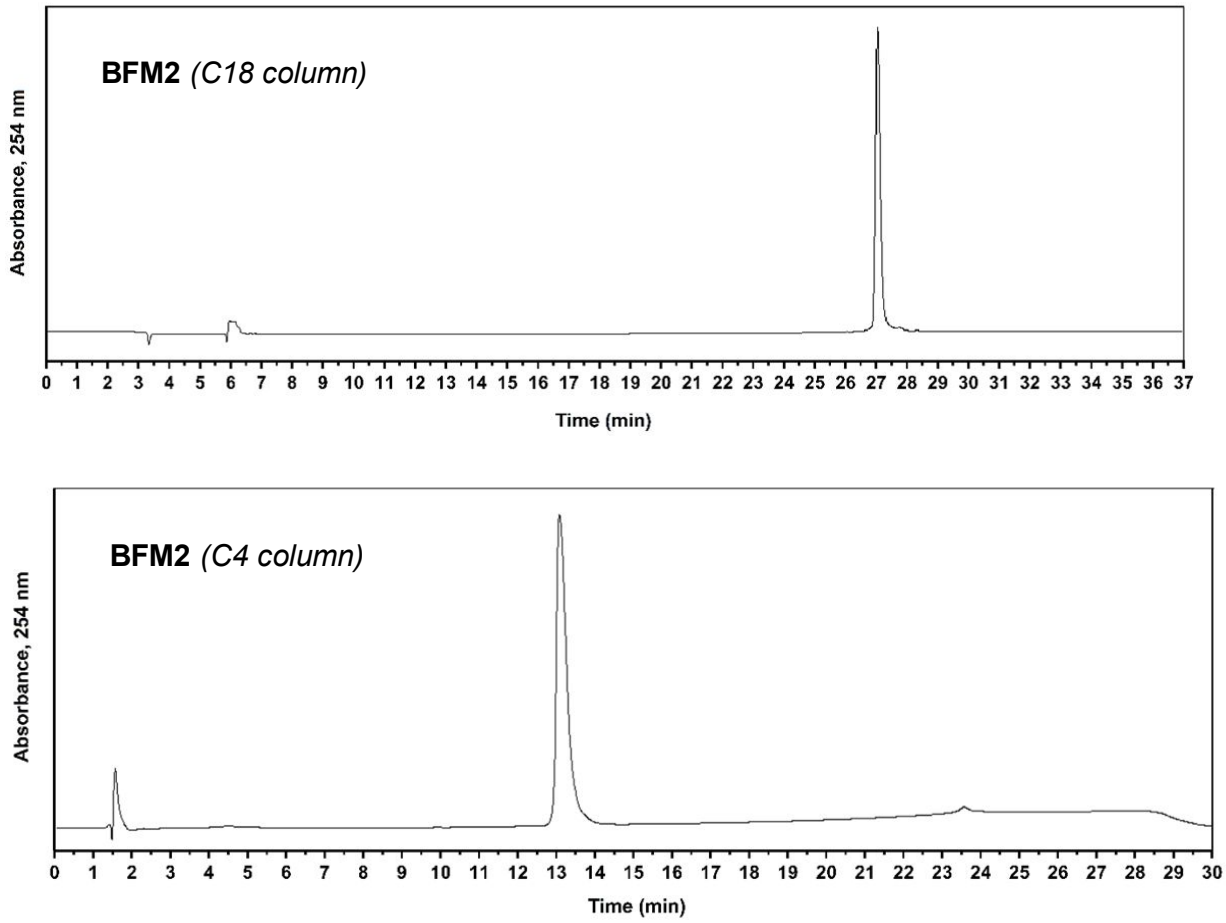
Synthesis of 21. To compound **15** (110 mg, 0.107 mmol, 1 equiv) was added a mixture containing TFA, TIS, and CH_2Cl_2 (50:3:100 ratio) (3 ml). The reaction was stirred at room temperature for 2 h and the solution was concentrated under reduced pressure. The residue was washed with CH_2Cl_2 (3x10 ml) and extracted with 25% MeOH/EtOAc. The combined organic extracts were concentrated under reduced pressure to afford compound **21** (60 mg, 91% yield); which was used directly in the next step. ESI-MS: Exact mas calcd for $C_{26}H_{41}N_5O_{10}S$ $[M+H]^+$ 616.26; $[M+Na]^+$ 638.24. Found: 616.5, 638.4].



BFM2 (9)

Synthesis of BFM2 (9). To a solution of **21** (45 mg, 0.073 mmol, 1 equiv) and VcMMAE (27 mg, 0.0205 mmol, 0.28 equiv) in anhydrous DMF (3 ml), was added triethylamine (0.1 ml). The

reaction mixture was stirred at room temperature for 5 h. The solution was concentrated under reduced pressure. The residue was washed with CH_2Cl_2 (3x10 ml) and then extracted with 20% MeOH/CH_2Cl_2. The combined organic extracts were concentrated under reduced pressure and purified by preparative HPLC to afford compound BFM2 (**9**) (26 mg, 66% yield); (95% purity by HPLC): t_R (column) (C18) = 27.2 min; t_R (C4) = 13.0 min; ESI-MS: Exact mas calcd for $C_{94}H_{146}N_{16}O_{25}S$ $[M+H]^+$ 1932.04; $[M+Na]^+$ 1954.03; $[M+2H]^+$ 966.56. [Found: 1932.5, 1954.5, 966.9].



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